The effects of neurotrophic factors on enteric ganglion cells in vitro

Thesis

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The Effects of Neurotrophic Factors on Enteric Ganglion Cells *in vitro*

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List of abbreviations

ABDS	Antibody diluting solution
ACh	Acetocholine
ANOVA	Analysis of variance
ART	Artemin
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
BN	Bombesin
CCK	Cholecystokinin
CGRP	Calcitonin gene related peptide
ChAT	Choline acetyltransferase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CREB	cAMP response binding protein
DNA	Deoxyribonucleic acid
ENK	Enkephalin
ENS	Enteric nervous system
ERK	Extracellular signal regulated kinase
FCS	Fetal calf serum
FKHRL	Forkhead transcription factor
GABA	Gamma aminobutyric acid
GAL	Galanin

GDNF	Glial cell-line derived neurotrophic factor
GDP	Guanine diphosphate
GFR	Glial cell-line derived neurotrophic factor receptor
GI	Gastrointestinal tract
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GTP	Guanine triphosphate
$HO^{\bullet-}$	Hydroxyl radical
H_2O_2	Hydrogen peroxide
5-HT	5-hydroxy tryptamine
IL	Interleukin
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
MAPK	Mitogen activated protein kinase
MEK	MAPK kinase
NADPH	Nicotinamide adenine dinucleotide phosphate diaphorase
NFP	Neurofilament protein
NGF	Nerve growth factor
NK	Neurokinin
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
NT-3	Neurotrophin 3
NTN	Neurturin

OD	Optical density
5-OHDA	6-hydroxydopamine
OSM	Oncostatin
PBS	Phosphate buffered saline
PACAP	Pituitary adenylyl cyclase
PGP 9.5	Protein gene product 9.5
PI	Propidium iodide
PI-3K	Phosphoinositide-3 kinase
PKB	Protein kinase B
PSP	Persephin
RNA	Ribonucleic acid
ROS	Radical oxygen species
SDS	Sodium dodecyl sulphate
SH	Src homology
SOD	Superoxide dismutase
SOM	Somatostatin
TGF	Transforming growth factor
TK	Tachykynin
VIP	Vasoactive intestinal peptide

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Abstract

The effects of neurotrophic factors on enteric ganglion cells were studied using variety of techniques. NT-3 was found to have protective effects on enteric neurons exposed to hydrogen peroxide. The second neurotrophic factor tested, GDNF, did not exhibit a similar, protective effect. Investigation of the effects of these factors on the levels of catalase and SOD Cu/Zn revealed that NT-3 does not affect levels of either enzyme after 12 and 36 hour incubation. In contrast to NT-3, GDNF was able to increase the levels of both enzymes, although this effect was observed only at the 36 hour time point. To investigate the mechanism of NT-3 induced protective effects, the signalling pathways initiated by NT-3 were also studied. PI-3K, an enzyme known to play a role in the survival responses initiated by trophic factors in many cell types was blocked using a specific inhibitor, LY294002. As a result, a dramatic decrease in cell numbers was observed in control as well as NT-3 and GDNF treated cultures. Inhibition of PI-3K also nullified the effects of trophic factors on the cells. Subsequently, the effects of NT-3 and combined NT-3/LY294002 treatment on the activity of several signalling proteins were examined. Control cultures showed steady, easily detectable levels of phosphorylated Akt, as well as total protein levels of bcl-2 and c-fos, which were independent of signalling initiated by addition of exogenous NT-3. In contrast, phosphorylation of MEK and ERK proteins was dependent upon exogenous NT-3. LY294002 treatment severely reduced the amount of phosphorylated Akt present in the cells. Additionally, the presence of phosphorylated MEK and ERK was observed at the point of NT-3 treatment, suggesting that in was independent of exogenous NT-3. Total protein levels of bcl-2 and c-fos proteins in the LY294002 treated cultures were elevated compared to controls.

Chapter 1

Introduction

Introduction

The enteric nervous system (ENS) is intrinsic to the digestive tract, and constitutes a part of the autonomic nervous system that innervates the gut. Although sympathetic and parasympathetic nervous systems also take part in the regulation of intestinal function the ENS is by far the most important and is unique in that it can function relatively independently of the control of the central nervous system (CNS). Functions of the ENS include the control of intestinal motility, secretion and blood flow. The ENS, like most of the peripheral nervous system, originates from the multipotent precursor cells of the neural crest that, after following specific pathways of migration, invade the entire gut. These enteric crest-derived cells respond to diffusible factors as well as extracellular matrix proteins generated by the microenvironment of the gut by differentiating into the cells of the enteric plexuses [Chalazonitis et al. 1996, Gershon et al. 1992]. It has been demonstrated that neurotrophic factors play an important role in the maturation process by promoting the differentiation of multipotent cells into enteric neurons and glia, and stimulating neurite outgrowth *in vitro* [Sternini et al. 1996, Chalazonitis et al. 1994,Yamamori et al. 1992]. In addition to their role in the development of the ENS, neurotrophic factors are also believed to be responsible for the plasticity of the nervous system in adults [Cowen et al. 1996].

This introductory chapter provides a general overview of the ENS, the neurotrophic factors acting on enteric neurons and a brief discussion of changes observed in the ENS during aging. Subsequent chapters contain detailed information describing the effects of oxidative stress on ENS (chapter 3), antioxidant enzymes involved in protecting enteric ganglion cells from free radicals (chapter 4) and finally, signalling pathways underlying actions of the neurotrophic factors on enteric neurons and glia (chapter 5).

1.1 Structure of the ENS.

The ENS consists of a number of interconnected networks, or plexuses. Two of those plexuses (the myenteric and submucous plexus) contain small groups of nerve cell bodies, called the enteric ganglia [Gershon et al. 1992, Costa et al. 1987]. (Fig.1.1).

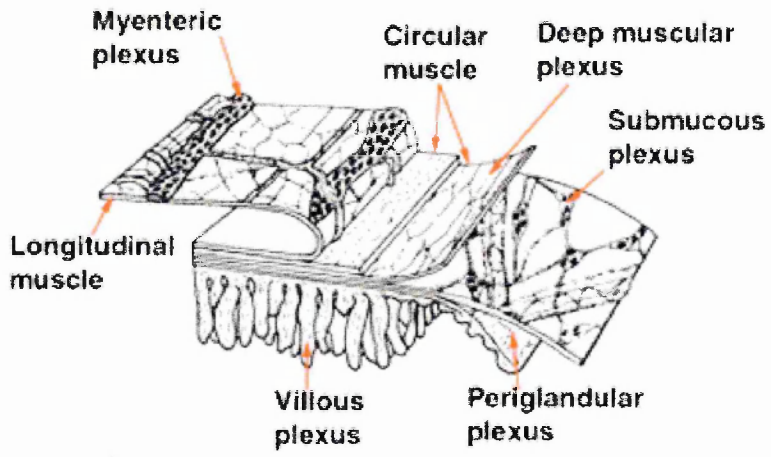


Figure 1.1 Structure of the ENS, Modified from [Costa et al. 1987]

The structure of the ENS is different from that of other peripheral nerves and ganglia. Collagen is excluded from the enteric ganglia and support for neuronal elements is provided by enteric glial cells, which share some similarities with astrocytes. Enteric glia differ from Schwann cells in that they do not form basal laminae and they ensheath axons, not individually but in groups [Costa et al. 1987].

The myenteric plexus is a network of nerve strands and small ganglia that lies in the plane between the external longitudinal and circular muscle coats of the intestine.
The network is continuous around the circumference of the gastrointestinal tract and along its length. The myenteric ganglia vary in size, shape and orientation from species to species and from one part of the intestine to another, but in any one area from a particular species the shape of the meshwork is quite characteristic and readily identified. Although the pattern is easily recognised in any one area, considerable variations in the sizes of ganglia are encountered. In the ileum of the guinea pig, myenteric ganglia range in size from 5 to 160 nerve cell bodies, with an average of 43 cell bodies per ganglion [Costa et al. 1987].
Within the submucosa there is a network of small ganglia and connecting strands which form the submucous plexus. The meshes of the submucous plexus are smaller than those of the myenteric plexus, the interconnecting strands are finer and the ganglia smaller. In the guinea pig small intestine, ganglia vary in size from single cells to groups of 30, with an average of eight, which is about one-fifth of the ganglion size in the myenteric plexus. The plexus is continuous along the circumference and along the length of the intestine. The ganglia often lie at two levels, one group being closer to the muscle, the other closer to the mucosa. [Wilson et al. 1981, Hoyle et al. 1989].

1.2 Neurotransmitters and neurons found in the ENS.

First attempts to classify enteric neurons date back to the late nineteenth century and the research of Dogiel, who identified at least three main classes of nerve cells based on their morphology (types I, II and III). [see Costa et al. 1989]. The first two cell types recognised by Dogiel have received the most attention, because they are easily detected in many species by a

variety of stains and their function has been elucidated. Dogiel type I neurons are often flattened in the plane of the ganglion in which they lie. A subset of Dogiel type I neurons has been identified as motor neurons. These cells have distinctly oval cell bodies, a single long process that is probably an axon, and, most characteristically, many paddle shaped lamellar dendrites projecting from the cell body. The cell bodies of Dogiel type II neurons are more spherical than those of type I cells and they give rise to several long processes, so that no single process can be distinguished as the axon. Research on the guinea pig identified a subpopulation of these neurons as the intrinsic primary afferent neurons [Kunze et al. 1999]. Type III cells have oval to spherical cell bodies, a single long axon, and many long thin dendrites that exhibit complex patterns of arborisation. Two additional cell types have been revealed by intracellular injections of the tracer: a filamentous cell with a single recognisable axon and even greater abundance of thin dendritic processes than the type II cell, and small simple neurons with few dendrites and a single recognisable axon. As many as ten different types of neurons have been described in the guinea pig myenteric plexus using ultrastructural criteria [Cook et al. 1976], however, it is not easy at this point to relate the ultrastructural classification of neurons to that of Dogiel.

In addition to classification based on cell morphology, enteric neurons have also been categorized according to their content of neurotransmitters. The ENS contains many neurotransmitters, and in many cases two or more neurotransmitters are present in one neuron [Furness et al. 2000, Legay et al. 1984]. There is good evidence in intestinal nerves for the presence of acetylcholine, cholecystokinin, dynorphin, met-enkephalin and leu-enkephalin, galanin, gastrin releasing peptide and a shorter form, neuropeptide Y, peptide HI, somatostatin, substances K, P and vasoactive intestinal peptide (Table 1.1).

Type of neuron	Proportion of total neuronal population	Chemical coding
Excitatory circular muscle motor neurons	12%	ChAT/TK/ENK/GABA
Inhibitory circular muscle motor neurons	16%	NOS/VIP/PACAP/ENK/NPY/GABA BN/NFP/Dynorphin
Excitatory longitudinal muscle motor neurons	25%	ChAT/Calretinin/TK
Inhibitory longitudinal muscle motor neurons	2%	NOS/VIP/GABA
Ascending interneurons (local reflex)	5%	ChAT/Calretinin/TK
Descending interneurons (secretomotor reflex)	2%	ChAT/5-HT
Descending interneurons (migrating myoelectric complex)	4%	ChAT/SOM
Myenteric intrinsic primary afferent neurons	26%	ChAT/Calbindin/TK/NK_3 receptor
Intestinofugal neurons	<1%	ChAT/BN/VIP/CCK/ENK
*Non cholinergic secretomotor/vasodilator neurons	45%	VIP/GAL
*Cholinergic secretomotor/vasodilator neurons	15%	ChAT/Calretinin/Dynorphin
*Cholinergic secretomotor (non vasodilator) neurons		
*Submucosal intrinsic primary afferent neurons	29%	ChAT/NPY/CCK/SOM/CGRP/Dynorphin
	11 %	ChAT/TK/Calbindin

Table 1.1 Types of neurons in the enteric nervous system and their neurotransmitters. Data based on the percentages of total neuronal population and neurotransmitters found in guinea pig small intestine. BN, bombesin; CCK, cholecystokinin; ChAT, choline acetyltransferase; CGRP, calcitonin gene related peptide; ENK, enkephalin; GABA, gamma amino butyric acid; GAL, galanin; 5-HT, 5-hydroxy-tryptamine; NFP, neurofilament protein; NK, neurokinin; NOS, nitric oxide synthase; NPY, neuropeptide Y; PACAP, pituitary adenylyl cyclase activation peptide; SOM, somatostatin; TK, tachykynin; VIP, vasoactive intestinal peptide. * Neurons located in submucous plexus. Adapted from [Furness et al. 2000].

The presence of serotonin in enteric neurons was confirmed by immunohistochemical staining [Legay et al. 1984]. One subset of enteric neurons, the enteric inhibitory neurons contain nitric oxide synthase and release nitric oxide. Although there is a good evidence that nitric oxide is a transmitter of these neurons, it is clear that it is not the sole transmitter, which was proved by knock out experiments in which gastrointestinal tract is little affected by the absence of nitric oxide synthase [Huang et al. 1994], and from incomplete block of transmission from enteric inhibitory neurons when nitric oxide synthase is blocked.

Different experiments indicate that most neurons contain more than one neurotransmitter [Costa et al. 1986, Furness et al. 1987]. For instance dynorphin, enkephalin and vasoactive intestinal peptide coexist in a significant proportion of type I neurons. A population of type III neurons contain cholecystokinin, calcitonin gene-related peptide, choline acetyltransferase (a marker of cholinergic neurons) and galanin. Some of the type I neurons contain nicotinamide adenine dinucleotide phosphate diaphorase (NADPH diaphorase), and a proportion of these neurons also contain neuropeptide Y [Gershon et al. 1986]. It is likely that other combinations of neurotransmitters also exist within enteric neurons.

In functional terms, the ENS comprises several types and many subtypes of neurons, some of which are listed in Table 1 and shown in Figure 1.2. Enteric motor neurons can be divided into excitatory neurons to the gut muscle, inhibitory neurons to gut muscle, secretomotor/vasodilator neurons, secretomotor neurons that are not vasodilator and neurons innervating the gastrin secreting endocrine cells of the stomach.

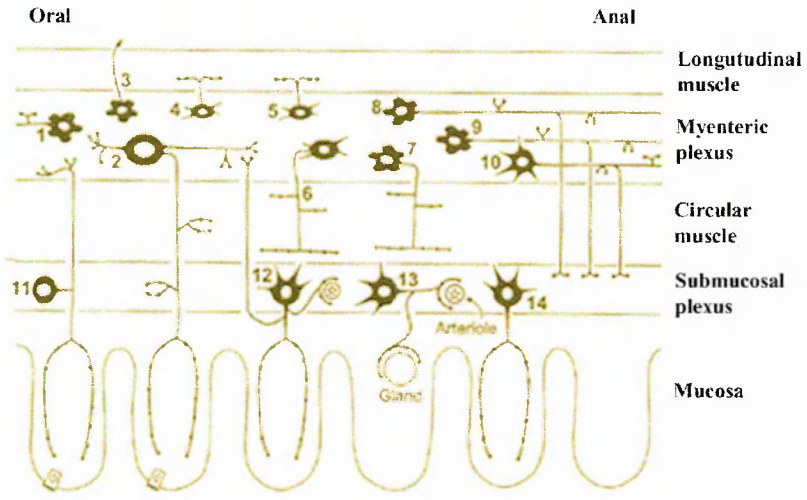


Figure 1.2 Types of neurons in the small intestine of the guinea pig.
1.Ascending interneuron, 2. Myenteric intrinsic primary afferent neuron, 3. Intestinofugal neuron, 4. Excitatory longitudinal muscle motor neuron 5. Inhibitory longitudinal muscle motor neuron, 6. Excitatory circular muscle motor neuron, 7. Inhibitory circular muscle motor neuron, 8. Descending interneuron (local reflex) 9. Descending interneuron (secretomotor reflex) 10. Descending interneuron (migrating myoelectric complex), 11. Submucosal intrinsic primary afferent neuron, 12. Non cholinergic secretomotor/vasodilator neuron, 13. Cholinergic secretomotor/vasodilator neuron, 14. Cholinergic secretomotor (non vasodilator) neuron. Modified from [Kunze et al. 1999]

Three types of extrinsic motor neurons directly innervate effectors in the gut: vagal motor neurons to the striated muscle of the esophagus, noradrenergic neurons that innervate ganglia and blood vessels in the gut as well as gut muscle and noradrenergic vasoconstrictor neurons that innervate arteries within the gut wall. All regions of the gut and each of the muscle layers receive an intrinsic excitatory innervation. It has been shown that excitatory transmission has a prominent muscarinic component, however there is a small residual excitation independent of it, most likely due to the release of tachykinins. Consistent with this observation, motor neurons are immunoreactive for both choline acetyltransferase (ChAT) and for tachykinins. The relative roles of tachykinins and ChAT are unequal since muscarinic antagonists substantially inhibit gastrointestinal motility *in vivo* [Galligan et al. 1986] whereas tachykinin antagonists have little effect. These observations suggest that acetylcholine is the primary

transmitter of excitatory muscle motor neurons. Inhibitory muscle motor neurons are the motor neurons that mediate the descending inhibitory reflexes and accommodation reflexes in the gut [Furness et al. 1987]. They contain nitric oxide (NO), which has been shown in many vertebrate classes including mammals. Although NO is readily detected, it is not the only neurotransmitter present in these neurons [Makhlouf et al. 1993]. The proof that NO is not the only neurotransmitter comes from gene knockout experiments, in which the gastrointestinal tract is affected only in small degree by the absence of nitric oxide synthase [Huang et al. 1994], and also from the incomplete block of transmission from enteric inhibitory neurons when NO synthase is blocked, or NO scavengers are used. There is some evidence that NO independent transmission might be attributed to ATP, VIP, PACAP and carbon monoxide. Electron microscopy studies suggest that all these different neurotransmitters come from the same neurons, since they detect only one population of inhibitory neurons, immunoreactive for NOS, VIP and PACAP [Costa et al. 1996]. There are four types of interneurons in the guinea pig intestine, one orally directed, and three types of anally directed. Orally directed neurons are cholinergic and like anally directed neurons form chains that extend along the gut. The three types of descending interneurons have the following chemical codings: ChAT/NOS/VIP, ChAT/SOM and ChAT/5-HT.

Studies of these neuronal classes have lead to the hypothesis that the first type, the ChAT/NOS/VIP neurons are involved in the conduction of migrating myoelectric complexes in the small intestine, and the ChAT/5-HT neurons are involved in secretomotor reflexes, but not directly in the motility reflexes [Pompolo et al. 1998]. The ChAT/SOM neurons are also distinctive in their morphology, having cell bodies with branching filamentous dendrites. Filamentous neurons with anally directed axons are not found in the distal colon, however in the colon there are filamentous neurons with orally directed processes.

Evidence for the existence of intrinsic primary afferent neurons in the gut comes from the studies that recorded reflexes in isolated intestine after extrinsic nerves supplying the intestine have been cut and their endings degenerated [Crema et al. 1970]. They were directly identified in the small intestine of the guinea pig, where these are Dogiel type II neurons [Bertrand et al. 1997]. Dogiel type II neurons with similar electrophysiological properties, projections and neurotransmitters have also been found in the large intestine of the guinea-pig and in the small intestine of the rat, which suggests that these neurons are also intrinsic sensory neurons.

The neurons responsible for controlling the balance of absorption and secretion of electrolytes are intrinsic secretomotor neurons. They are controlled through local reflex circuits, which in turn remain under control of the central nervous system. To date, two types of intestinal secretomotor neurons, cholinergic and non-cholinergic have been identified. In addition, release from the ends of intrinsic primary neurons in the mucosa may also have secretomotor effects. The non-cholinergic neurons appear to mediate most of the local reflex responses and use VIP or related peptide as their main neurotransmitter [Reddix et al. 1994].
In the guinea-pig small intestine there are two types of cholinergic secretomotor neurons, those that also contain NPY, and those that contain calretinin. There is also a single class of non-cholinergic secretomotor neurons which is immunoreactive for VIP. The ACh/calretinin neurons preferentially innervate the glands at the base of the mucosa and have collaterals to submucosal arterioles, whereas the ACh/NPY neurons do not innervate the arterioles. The presence of three classes of secretomotor neurons, two of which also provide vasodilator collaterals could provide a mechanism to balance secretion and vasodilation depending on digestive state.

Although the majority of ENS research has concentrated on guinea pig, there are also some studies addressing the questions of neurotransmitters and neuronal circuitry present in rat ENS, which shares many similarities with guinea pig including similar major classes of neurons [Mann et al. 1998].

1.3 Development of the enteric nervous system

The ENS is derived from the neural crest. Prospective enteric neural crest cells emigrate from two main regions of the neural tube: the vagal and sacral regions. Cells from a third region that lies within the truncal crest colonize only the rostral foregut, mainly the esophagus and cardiac stomach [Durbec et al. 1996]. Vagal-derived progenitors of the ENS, which give rise to the majority of neurons and glia of the enteric ganglia, enter the foregut mesenchyme and migrate in an anteroposterior direction, colonising the entire length of the gut [Taraviras et al. 1999]. Studies conducted on mouse embryos have suggested that the enteric component of the vagal neural crest generates two distinct lineages: first the sympathoenteric lineage which contributes to the formation of the enteric ganglia throughout the entire length of the gut and the ganglia of the sympathetic chain, and second the sympathoadrenal lineage, which generates progeny that colonise primarily the enteric ganglia of the foregut: esophagus and stomach [Durbec et al. 1996]. In addition to the vagal region, neural crest cells emigrating from the sacral crest migrate into the gut and contribute to the formation of the postumbilical ENS [Pomeranz et al. 1991]. Studies performed on mouse embryos show that initially sacral neural crest cells migrate in a caudal to rostral direction and form the ganglia along the dorsal part of the hindgut and midgut up to the opening of the bile and pancreatic ducts. Later on the neural crest-derived cells enter the hindgut after which their number increases significantly,

reaching a maximum between E10-12. The vast majority of the sacral neural crest cells are restricted to the colorectum, whereas far fewer sacral cells are present in the ceca and in the postumbilical intestine [Burns et al. 1998].

The fact that the bowel is colonised by cells from only two regions of the neural crest raises the possibility that the precursors in these regions might be committed to enteric lineages. Many experiments, however, indicate that it is unlikely that either vagal or sacral crest cells are committed to be enteric neurons or glial cells before they leave the neural crest. Behaviour of cells in quail-chick chimeras suggests that the fate of crest-derived cells depends on their location in the host embryo rather than on the site from which the grafts were obtained. Moreover, within the gut the quail's crest-derived cells, despite their truncal origin, gave rise to bowel-appropriate neuronal phenotypes, which include cholinergic, peptidergic and serotonergic neurons. Serotonergic neurons are not formed by truncal crest-derived cells *in situ,* which suggests an influence of the enteric microenvironment on neural differentiation [Gershon et al. 1992]. The implication of these data is that crest-derived cells migrate through embryos along defined pathways, thus even cells from the wrong region of the crest will find the gut if placed on a path that leads them there. The terminally differentiated phenotype expressed by cells of neural crest origin, moreover, is not determined before these cells begin to migrate. Phenotypic expression can be thus influenced by signals encountered by crest-derived cells, either along the migration pathway, or at the site of terminal differentiation. Among the molecules having such an effect on migrating neuronal precursor cells are neurotrophic factors, shown to play a critical role in the formation and development of the ENS [Chalazonitis et al. 1998].

1.4 Neurotrophic factors

Trophic factors include substances that have effects on cell differentiation, survival, phenotypic expression and plasticity, as well as on cell growth, for example neurite extension. Neurotrophic factors, a subset of trophic factors acting on neural tissue have been defined in many ways, from very restrictive to general. Restrictive definitions are based on the research conducted on nerve growth factor, and include only the specific aspects of the developmental events, such as cell survival and neurite extension. This restrictive definition of a neurotrophic substance parallels the restrictive definition of and requirements for a neurotransmitter, which were based on experimental paradigms developed for acetylocholine. As more putative neurotransmitters and neurotrophic factors have been discovered and analysed, it has become clear that broader definitions of each group are necessary to capture the range of actions of these neuromessengers. One of the modern definitions of neurotrophic factors describes them as endogenous, soluble proteins regulating survival, growth, morphological plasticity or synthesis of proteins for differentiated functions of neurons. This definition combines aspects of a functional and physical nature and is compatible with the view that growth factors are multifunctional [Loughin et al. 1993]. Neurotrophic factors perform diverse functions in both the developing and mature nervous system, ranging from promoting survival and differentiation to regulation of neuronal phenotype and maintenance of mature neurons [Chalazonitis et al. 1996 and 1994, Cowen et al. 1998]. Many neurotrophic factors have been shown to influence enteric neurons, most of them belong to one of three main families: the neurotrophins, the glial cell-line derived neurotrophic factors (GDNF) family and the neuropoietic cytokines.

1.4.1 The GDNF family of neurotrophic factors

Glial cell line-derived neurotrophic factor (GDNF), and the related factors neurturin (NTN), artemin (ART), and the recently identified member of this family, persephin (PSP), form the GDNF family of neurotrophic factors. They form a subgroup in the transforming growth factor-β (TGF-β) superfamily. These factors are basic, dimeric, secretory proteins that share cysteine knot structure. GDNF, first identified as a trophic factor for midbrain dopaminergic neurons, promotes the survival of many types of neurons including subpopulations of peripheral autonomic and sensory, as well as central motor, dopaminergic and noradrenergic neurons [Henderson et al. 1994, Tomac et al. 1995]. NTN was cloned as a survival factor for sympathetic neurons, after which ART and PSP could be identified on the basis of sequence homology. NTN and ART have many neurotrophic effects similar to GDNF, they all support survival of peripheral sympathetic and sensory neurons as well as midbrain dopamine neurons. PSP is expressed at low levels in most tissues and supports central nervous system dopaminergic and motor neurons, but not peripheral neurons [Milbrandt et al. 1998].

All members of the GDNF family employ the c-Ret receptor to initiate signalling cascades. c-Ret was originally identified as an oncogene activated by DNA rearrangement, causing human papillary thyroid carcinoma, endocrine neoplasia and Hischsprung's disease [Carlson et al. 1994, Romeo et al. 1994]. The RET proto oncogene encodes a receptor tyrosine kinase with a cadherin related motif and a cysteine rich domain in the extracellular domain (Fig. 1.3). Three c-Ret isoforms of different length, generated by alternative splicing of the 3' region have been cloned so far [Lee et al. 2003]. In rodent embryonic and adult tissues, c-Ret is highly expressed in peripheral enteric, sympathetic and sensory neurons as well as central motor, dopaminergic and noradrenergic neurons [Tsuzuki et al. 1995]. In addition to the nervous

system, c-Ret expression has been reported in the excretory system such as mesonephric duct and branching ureteric bud during embryogenesis. In addition to the c-Ret receptor complex necessary to initiate signalling, ligand binding also requires a GPI-anchored component, GFR α which is a GDNF-family coreceptor. Four GFRα coreceptors have been identified to date. All four known GDNF family members have their own preferred coreceptor. GFRα-1 shows specificity towards GDNF, GFRα-2 preferentially binds NTN while GFRα-3 binds ART. The PSP receptor, GFRα-4 has been found only in chicken. The sequence of GFRα-4 shows more similarity to GFRα-1 and GFRα-2 than to GFRα-3 [Ariaksinen et al. 1999]. Although coreceptors show preferential binding to their ligands, alternative ligand-receptor binding has been shown to occur, for example GDNF can bind to GFRα-2 and GFRα-3 in the presence of c-Ret, and ART and NTN can bind to GFRα-1 (Fig. 1.4) [Baloh et al. 1997, Jing et al. 1997]. Expression patterns of both GDNF and c-Ret suggest that they could

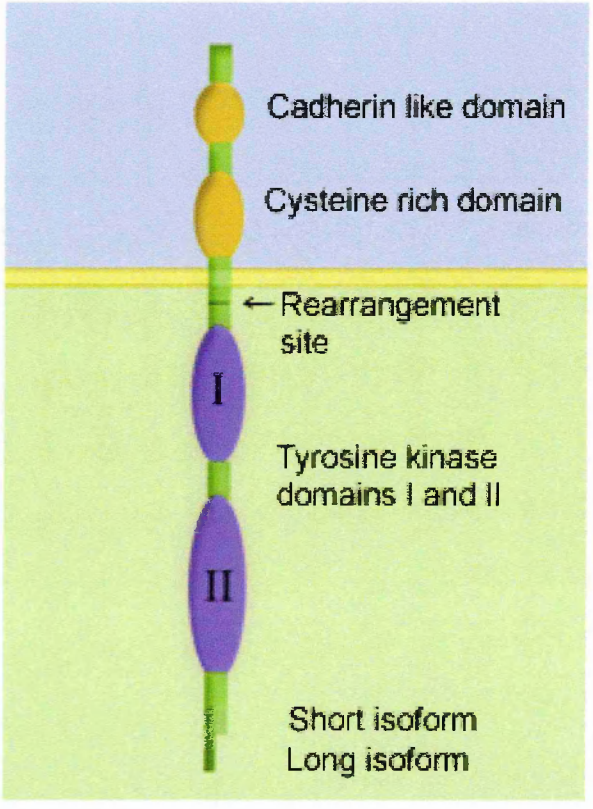


Figure 1.3 Schematic representation of the domains of Ret receptor tyrosine kinase.

be involved in the development of the ENS, and this has been confirmed by experiments using GDNF/c-Ret knockout mice. These animals showed lack of enteric neurons as well as renal agenesis or dysgenesis, causing death soon after birth. A similar phenotype has been observed in GFRα-1 knockouts. [Durbec et al. 1996]. In contrast to the lethal phenotype of GFNF and GFRα-1 mutants, mice lacking NTN or GFRα-2 are born normally, and show no developmental abnormalities. However, the ENS in these animals exhibits a decreased plexus density, which correlates with functional deficit in contractile rhythm and activity [Rossi et al. 1999]. Considering the fact that GFRα-2 expression is weak in the embryonic bowel and peaks several weeks after birth [Golden et al. 1999] which is the reverse of the expression pattern of GFRα-1, it seems likely that NTN/ GFRα-2 signalling is important for later developmental events, and for the maintenance of the mature ENS.

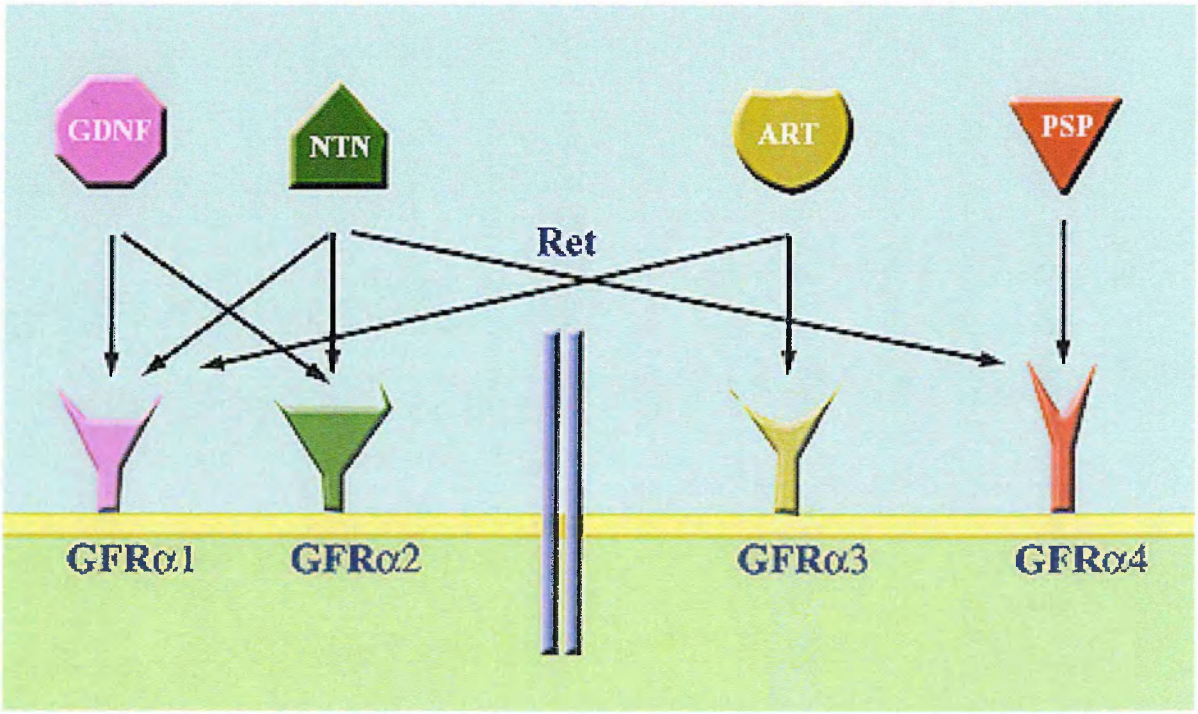


Figure 1.4 The GDNF family of neurotrophic factors and their preferred receptors. Also shown are possible alternative ligand/receptor interaction.

1.4.2 Neurotrophins

Nerve growth factor (NGF) was the first identified member of the neurotrophin family, discovered as early as the 1940's [Levi-Montalcini et al. 1995]. The second member of the neurotrophin family, brain derived neurotrophic factor (BDNF) was discovered forty years later, in the 1980's, with the cloning of the remaining molecules belonging to this family, NT-3 and NT-4/5 soon after [Bothwell et al. 1995]. All neurotrophins are small, basic proteins, active as non covalently linked dimers. Neurotrophins are secretory proteins, with cleavable leader sequences, followed by a pro-sequence. Cleavage of the pro-sequence at the consensus site common for all neurotrophins yields the biologically active molecules. All members of neurotrophin family share about 50% protein sequence homology, including 6 cysteine residues necessary for proper folding of the mature protein (Fig. 1.5). With the exception of the NT-4/5, neurotrophins are highly conserved between species.

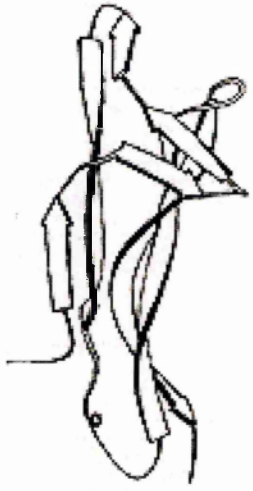


Figure 1.5 Ribbon model of the structure of NGF protein monomer.

Neurotrophins bind to two types of receptors, trks and p75. Trk receptors are transmembrane tyrosine kinase receptors that bind specific members of the neurotrophin family. The first

discovered was trk A, observed as a transforming oncogene in colon carcinoma [Martin et al. 1986].

The trk family members are highly related, particularly within their tyrosine kinase domains. Each of the mammalian trk family members has a signal peptide of about 32 amino acids, a glycosylated extracellular domain, a single short transmembrane domain and an intracellular domain containing tyrosine kinase activity. Molecular and biochemical analyses have revealed the presence of alternative splice variants of the trks. Of these the most prevalent are truncated forms of trk B and trk C, which are expressed at high levels in the mature brain [Middlemas et al. 1991]. These truncations are intracellular and include the kinase domain, rendering the receptors non-functional with respect to tyrosine kinase activity. Trk receptors show specific binding of respective members of neurotrophins, trk A preferentially binds NGF, trk B BDNF and NT-4/5, while trk C shows specificity towards NT-3.

In addition to the trk tyrosine kinase receptors which show differences in specificity for particular neurotrophins, the p75 receptor binds all members of the neurotrophin family [Chao et al. 1994]. p75 is a transmembrane glycoprotein belonging to the TNF/Fas/CD40 superfamily of receptors. Specific conserved structural elements of this receptor family shared by p75 include cysteine rich repeats in the extracellular domain, containing the binding site for NGF [Yan et al. 1991], and an intracellular death domain [Liepnish et al. 1997]. No alternatively spliced forms of p75 mRNA have been detected, however a soluble form of p75 capable of binding neurotrophins has been described [Zupan et al. 1989].

p75 can interact with the different Trk receptors to modulate neurotrophin actions. For example coexpression of p75 with Trk A increases high affinity binding of NGF and

influences the ability of NGF to stimulate Trk A autophosphorylation in PC12 cells [Barker et al. 1994]. Experiments involving use of antisense nucleotides against p75 and p75 null mice demonstrate that peripheral sympathetic and sensory neurons also display reduced sensitivity to NGF without p75 [Davies et al. 1993]. In addition to modifying the binding of neurotrophins to Trk receptors, p75 may contribute to the ability of Trk receptors to discriminate the preferred ligand from the other neurotrophins. When Trk receptors are expressed heterologously in fibroblast cell lines which do not express p75 the responsivity to different neurotrophins in less restricted than in neurons, for example NIH 3T3 cells expressing Trk A respond to NT3 as well as NGF, but in PC12 cells and neurons expressing p75 as well as Trk A the response was restricted to NGF [Ip et al. 1993]. When p75 levels were lowered in PC12 cells they were increasingly responsive to NT-3, confirming the role of p75 in restricting ligand specificity. In addition to the ability to modulate the function of Trk receptors, p75 can also initiate cell death when expressed in the absence of Trk's. In neuronal cell lines expression of p75, in the absence of a coexpressed Trk receptor has been shown to mediate induction of cell death by NGF [Rabizadeh et al. 1993]. Similarly in primary sympathetic neurons which express p75 and Trk A, but not Trk B, BDNF treatment leads to apoptosis, indicating that interaction of a neurotrophin with p75 in the absence of Trk signalling leads to cell death [Lee et al. 2001].

Mice null for NGF or its receptor, TrkA, as well as brain derived neurotrophic factor (BDNF) showed no losses in populations of CNS neurons. Part of the reason for that might be much more redundant support received by CNS neurons, due to their more numerous connections. However, the role of neurotrophins in the development of the ENS is much more pronounced. Members of the neurotrophin family, in particular NT-3 have been demonstrated to play a role in the development of enteric neurons and glial cells [Chalazonitis et al. 1996], which is

suggested by the observation that Trk C, the receptor specific for NT-3 is expressed by crest derived cells isolated from the fetal bowel [Chalazonitis et al. 1994]. However, NT-3 is most likely not the only factor acting during the development of the ENS, as demonstrated by the use of transgenic mice null for NT-3 or Trk C, where most of the neuronal structures expressing these genes has shown no abnormalities [Klein et al. 1994].

1.4.3 Neuropoietic cytokines

The amino acid sequences of rat, rabbit and human CNTF show the features of a cytosolic protein with no signal peptide, no consensus sequences for glycosylation and only one free cysteine residue at position 17. Because of that, it is not secreted but found in the cytoplasm of the cells such as astrocytes and schwann cells [Rende et al. 1991, Rudge et al. 1992]. CNTF was initially identified by its ability to support the survival of parasympathetic neurons of the chick ciliary ganglion *in vitro* [Adler et al. 1979]. CNTF is structurally and functionally related to members of a family of cytokines that include leukemia inhibitory factor (LIF), interleukin 6 (IL-6), interleukin 11 (IL-11) and oncostatin M (OSM). CNTF was predicted to share common structural characteristics of most of these cytokines, which comprises a four helix bundle. Elucidation of the CNTF receptor structure supports the notion that CNTF is related to LIF, IL-6 and OSM, due to the fact that all four of these cytokines share the use of similar receptor components, that is LIFRβ and gp 130 [Davis et al. 1993]. Some of these factors, including CNTF also require a specificity determining α receptor component (Fig. 1.6). The CNTF α component (CNTFR α) is homologous to the α component used by IL-6, but in contrast to IL-6Rα CNTFR α is generally restricted to the nervous system in its

expression, thus largely limiting the actions of CNTF to neuronal cells. CNTFR α is also expressed in fetal bowel [Rothman et al. 1994] suggesting it might play a role in the development of the ENS, which was confirmed by experiments disrupting expression of CNTFR α: such mutation is lethal and mice carrying it die soon after birth due to the lack of enteric motor neurons [Gershon et al. 1997, Li et al. 1995]. In addition, mice with targeted mutations that disrupt expression of LIFR β also die shortly after birth [DeChiara et al. 1995]. Since the ENS has not been found to be abnormal in animals that lack CNTF or LIF, it is likely that the bowel expresses a ligand other than CNTF that is capable of activating the CNTF receptor complex by binding to CNTFR α [Li et al. 1995].

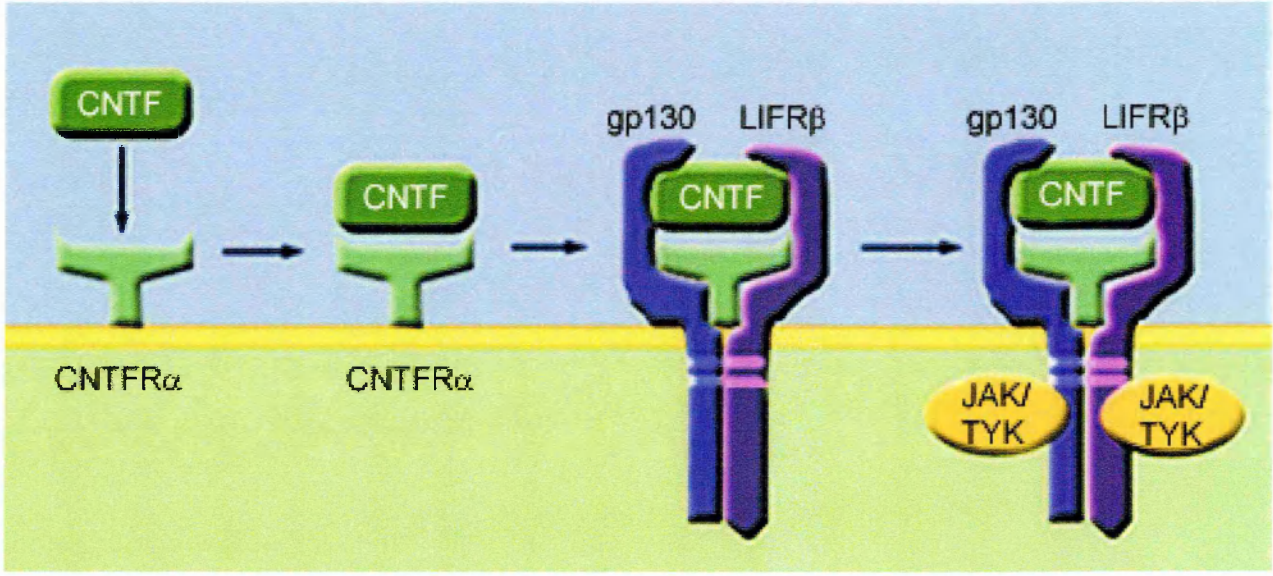


Figure 1.6 Formation of the CNTF signalling complex. First step requires binding of CNTF to its specific receptor, CNTFRα, which then binds to gp130, and finally to LIFRβ receptors, which initiates the signalling cascade.

Even though CNTF and LIF may not be essential for the development of the ENS, their ability to activate the CNTF receptor complex makes it possible to use these ligands to analyse the role of the tripartite CNTF receptor in the development of enteric neurons and glia [Ip et al.

1992]. Research on the role of ciliary neurotrophic factor (CNTF) has shown that administration of CNTF increases the survival of hippocampal neurons *in vitro* [Ip et al. 1991]; it also prevents degeneration of dopaminergic neurons in CNS [Hagg et al. 1993].

There is evidence suggesting that neuropoietic cytokines collaborate with NT-3 in the development of at least a subset of enteric neurons. The progenitors that colonize the bowel below the rostral foregut depend for their survival and subsequent development upon activation of the c-Ret receptor complex by GDNF (Fig. 1.7) [Trupp et al. 1996].

Two distinct lineages of neurons arise from the early GDNF/c-Ret dependent precursors. One of these is transiently catecholaminergic and dependent on expression of the mash-1 (mammalian homologue of achaete-scute complex) gene, which promotes differentiation of neuronal precursor cells [Guilemot et al. 1993]. This lineage shares properties of sympathoadrenal precursors and is born early, giving rise to all of serotonergic neurons of the ENS [Blaugrund et al. 1996].

Both NT-3 and CNTF/LIF have been demonstrated to affect the *in vitro* development of sympathoadrenal cells [Patterson et al. 1992]. Moreover, the effects of cytokines on sympathoadrenal cells are modified when they are applied in combination with a neurotrophin [Ip et al. 1994]. Except their effects on developing neurons, neurotrophic factors are also known to participate in the maintenance and protection of adult and aging neuronal populations.

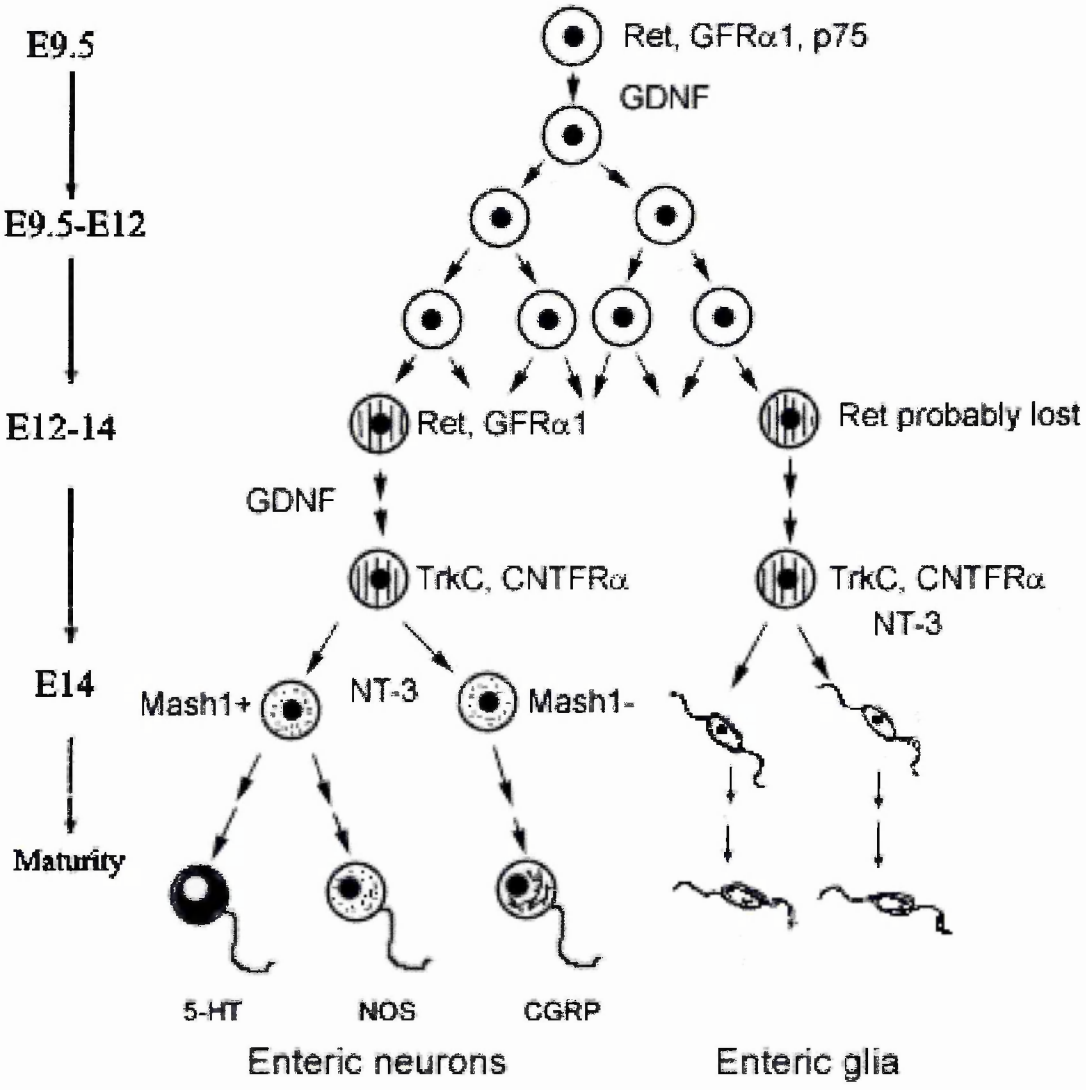


Figure 1.7 Model of the neurotrophic factor dependence of enteric precursor cells during the development of the enteric nervous system. Selected markers specific for the cells at given developmental stage are also included. Modified from [Chalazonitis et al. 1998]

The most common free radicals produced in mitochondria include superoxide anions, hydrogen peroxide and hydroxyl radicals. Many cellular components are susceptible to free radical damage, these include cellular membranes. For example, free radicals react with polyunsaturated fatty acids to form lipid peroxides which in turn decompose to give rise, in several reactions, to mutagenic malondialdehyde [Lippman et al. 1983]. Lipid peroxides have been shown to produce an irreversible impairment of membrane fluidity and elasticity, which could lead to rupture of cells. Such changes are likely to have most significant effects on post mitotic cells such as neurons. The breakdown products of lipid peroxidation are also belived to contribute to the production of lipofuscin, a brown pigment that accumulates with age and which appears to correlate with the age of animal cells. Again, the build up of lipofuscin is particularly noticeable in postmitotic cells and it has been shown that by the age of 90 years up to 7% of the intracellular volume of a human myocardial cell may be occupied by this pigment [Brody et al. 1995]. Free radical activity has also been shown to oxidise and cross link proteins including enzymes and connective tissue. In fact, oxidised proteins in an old animal may represent up to 50% of the total cellular protein content and this is likely to be a major reason for the observed drop in catalytic activity of many enzymes correlated with age [Stadtman et al. 1995]. Another type of molecule that is highly susceptible to free radical damage is DNA. Most common effects of free radicals on nucleic acids include base deletion and strand breakage. Although repair systems correct most of the damage, oxidative DNA lesions nevertheless accumulate with age especially at the later stages of life due to the lowered efficiency of DNA repair systems [Rao et al. 1994, Chen et al. 2002], contributing to the aging process.

The organelles by far most affected by free radical damage are mitochondria. While they use about 90% of the total amount of oxygen consumed by the cell, their genetic material,

unprotected by histones is highly susceptible to free radical damage. That and the fact that mitochondrial DNA repair systems are less effective than those present in the nucleus [Bohr et al. 2002] results in the far greater number of oxidative lesions occurring to the mitochondrial DNA, about ten times more than to nuclear DNA.

The rate at which mitochondrial DNA accumulates damage is also much faster than in the nucleus, especially in postmitotic cells [Bhaskar et al. 2000]. As a consequence, ATP production and mitochondrial turnover gradually decreases during the life of the cell. It has also been demonstrated that the mitochondria of older animals produce significantly higher levels of reactive oxygen species than these of younger animals [Ross et al. 2000]. Thus, it can be hypothesised that in an escalating process, damaged mitochondria leak more free radicals, contributing to the increasing amount of damage to the cell and themselves. As an outcome, one might hypothesise that energy available to the cell would be depleted, and tissues would degenerate, which indeed is the case in aged animals.

The only way to slow down the onset of age associated changes demonstrated so far is caloric restriction: the reduction of food intake without loss of essential nutrients and minerals. This was first shown by McCay [McCay et al. 1935], who reared rats on a diet that reduced the amount of calories by about 30% compared to *ad libitum* fed animals. The results showed that males introduced to this diet two weeks after weaning experienced a significant extension of mean life span compared to controls. Maximum life span was also significantly increased with the longest lived calorically restricted rat living for 1465 days compared to 969 days for the oldest control. Another striking feature was the healthy demeanour of the caloric restricted rats. At the age of 1000 days, at which point all the control animals had died, the calorically

restricted rats were still highly active and displayed the morphology of much younger animals. Despite its importance, the mechanisms underlying the anti-aging effects of caloric reduction are still poorly understood. One proposal has been that food restriction retards ageing by slowing down the basal metabolic rate [McCarter et al. 1985]. However, this theory has not gained much support. For example, direct measurements of oxygen intake over 24 hours period has demonstrated that food restriction does not decrease the basal metabolic rate per unit of body mass [McCarter et al. 1985]. Examination of food intake records over the life span leads to a similar conclusion and if anything shows that the total lifetime consumption of calories in underfed animals is greater than normal [Masoro et al. 1991]. A more convincing metabolic explanation is that food restriction causes a major reorganisation of energy utilisation [Feuers et al. 1995]. For example, food deprivation has been shown to produce a lowering of average body temperature and a shift away from fat production towards glucose synthesis. It has also been found that calorically restricted animals have a normal metabolic rate prior to feeding but a higher one just after eating. In addition, a wide range of enzymes is altered by dietary restriction including those in the liver participating in the drug metabolism and elimination, as well as energy metabolism [Hagopian et al. 2002]. There is considerable evidence however to support the idea that dietary restriction might be acting to reduce the formation of free radicals. For example, it has been shown that age-related reductions in the antioxidant enzymes are partially prevented by dietary restriction, and this is also accompanied by lower levels of superoxide and hydroxyl radicals along with decreased lipid peroxidation of liver mitochondrial and microsomal membranes [Rao et al. 1990]. This may be, in part under genetic control as reduced food intake modulates the expression of a wide range of genes including those controlling antioxidant enzymes [Rao et al. 1990]. Additionally, food restriction protects against the age-related decline of mitochondria to oxidise malondialdehyde, which is a highly potent source of free radicals.

In conclusion, increased antioxidant defence systems coupled with lower production of free radicals is likely to be the mechanism by which caloric restriction exerts its effects.

1.6 Age-related changes in the enteric nervous system

There is compelling evidence for an increased incidence of gastrointestinal tract disorders with advanced age [Bannister et al. 1987, Madsen et al. 1992]. It has been reported that the rate of colonic transit is slowed in elderly humans [Madsen et al. 1992], a similar finding has also been made in old rats [McDougal et al. 1984]. An age-related reduction in both frequency and amplitude of the peristaltic wave has also been reported in elderly patients [Horowitz et al. 1984], as well as decrease in digestion and absorption of food and immune function. Since the ENS is responsible for coordinating and integrating the motility of the gut, it has been extensively studied in order to establish its role in the motility disorders in the elderly [Gomes et al. 1997]. In contrast to neurons in other autonomic nerve groups, quantitative studies of cell numbers within the myenteric plexus have consistently demonstrated extensive losses in the oesophagus, small and large intestine of old guinea pigs and rats [Gabella et al. 1989, Santer et al. 1988]. The submucosal plexus is affected by age in similar manner, as was shown by the neuronal loss in aged compared to young mice [El-Salhy et al. 1999] although the number of ganglia present in the submucous plexus did not decrease [El-Salhy et al. 1999]. Age-dependent changes in the intestine are not restricted to neuronal numbers. Isolated organ bath experiments on segments of intestine taken from aged rats have revealed significant reduction in the cholinergic component of neuromuscular transmission [Nowak et al. 1990].

Immunohistochemical studies have demonstrated a reduced density of peptidergic fibres in the gastrointestinal tract of aged rats [Barker et al. 1991]. The density of extrinsic fibres to the myenteric plexus, projecting from the coeliac superior mesenteric ganglion and containing noradrenaline also significantly declines with age [Barker et al. 1991]. Reports concerning age dependent changes in the activity of antioxidant enzymes in the gastrointestinal tract are somewhat contradictory and vary from study to study, however it was found that activities of at least some of the antioxidant enzymes are decreased in aged animals [Xia et al. 1995] In general, most of the overall changes observed in the ENS during aging stems from the gradual loss of neurons, or at least some their populations. In multicellular organisms, the aging process is usually characterized by a decline of function and increased rate of pathological conditions. To identify the molecular events associated with aging in mammals, several laboratories have used the microarray expression analysis. To date, these studies have provided insight into global, age-associated changes in gene expression in mouse skeletal muscle, human skin fibroblasts and mouse liver [Lee et al. 2000, Han et al. 2000], as well as duodenum and colon in rats. What is interesting, is that the data reveal few similarities in the age associated changes in gene expression patterns among these tissues which suggests that different mechanisms may underlie the aging process in different cell types. This observation highlights the complexity and intricacy of aging process, as well as its dependence on the particular cellular background.

1.7 Aims

Neurotrophic factors have been shown to reduce free radical production in the neurons *in vitro* [Dougan et al. 1997] (T.Cowen, personal communication), possibly by affecting the antioxidant enzymes present in the cells. For that reason, neurotrophic factors might be useful in counteracting gradual decline in numbers of enteric ganglion cells occurring with age, especially since ROS induced damage to the cellular components has been implicated in this process.

Considering these facts, it is possible that neurotrophic factors participate in the mechanisms evoked by caloric restriction, which result in reduced free radical damage, and extend the life span of enteric ganglion cells.

This study was undertaken in order to investigate the possible roles of NT-3 and GDNF in the survival of enteric ganglion cells exposed to free radical damage. The effects of these factors on cultured enteric ganglia are described in first chapter, the second chapter focuses on the possible effects of NT-3 and GDNF on the protein levels of antioxidant enzymes. The final chapter examines signalling cascades underlying the effects NT-3 described in the previous chapters.

Chapter 2

Materials and Methods

2.1 Primary cultures of enteric ganglion calls and factor treatment

Enteric ganglion cell cultures were prepared described in [Saffrey et al. 2000]. Briefly, small intestines of 7 day old Sprague-Dawley rats were harvested and placed in Hanks balanced salt solution (HBSS, Sigma) containing gentamycin and metronidazole. After removing the mesentery, the muscularis externa with embedded enteric ganglion cells was separated by peeling from the rest of the gut and incubated for 30 minutes in collagenase type II (1mg/ml in HBSS containing 10 μg/ml DNase) at 37°C. Subsequently the tissue was vortexed and incubated with fresh enzyme for another 30 minutes. Freed pieces of plexus were collected under a dissecting microscope, placed in trypsin-EDTA solution (Sigma), incubated for 15 minutes and dispersed by passage through a 25 gauge needle to obtain a single cell suspension. Cells were seeded in 150 μl of 199 medium with N1 supplements (N 199, Sigma) containing 10% fetal calf serum (FCS, Sigma) at 2 X 10^4 cells per 13mm diameter glass coverslip coated with poly L lysine. After 1 hour incubation at 37°C, 2.5 % CO_2 a further 850 μl of 199 N1 medium without FCS was added. Cultures designed for preparation of cell lysates subsequently used in western blots were seeded in 1ml of 199 medium with N1 supplements and FCS at 7 X 10^5 cells per 30mm diameter glass coverslip, covered with poly L lysine; after 1 hour incubation medium was replaced with 2 ml of 199 medium with N1 supplements without FCS. 16 hours later medium was removed and replaced with serum-free 199 N1. Cells were supplemented with desired concentrations of appropriate factors 24 hours after plating.

2.2 Hydrogen peroxide exposure

Dilutions of hydrogen peroxide (Sigma) were made fresh from 30% stock solution into HBSS prior to each experiment. Cultures of enteric ganglion cells incubated with NT-3 or GDNF were exposed to the desired concentrations of H_2O_2, and subsequently incubated prior to the cell viability assay for 6 hours (MTS assay) or 4 hours (Bis-benzimide/propidium iodide staining) at 37°C, 2.5% CO_2.

2.3 MTS viability assay

Viability of the cells exposed to H_2O_2 was assayed using the CellTiter96^R kit (Promega). Assay is based on a conversion of tetrazolium compound by mitochondrial enzymes to formazan, absorbing at 490nm. The absorbance values obtained in this assay correspond to the number of live mitochondria, and indirectly the number of live cells present in the cultures. In the experimental design used in this work the number of cells was constant in each experiment, which allows relating the absorbance values obtained in the assay to the number of viable cells in the cultures. Assay was performed as follows: 6 hours after exposure to H_2O_2, the growth medium of the cultures was replaced with HBSS without phenol red (Sigma), and MTS reagent was added. After 4 hours incubation at 37°C supernatants were collected, and their absorbance was measured at 492 nm. Experiments were repeated 3 times; at least 4-6 replicate treatments were used for each condition, in each experiment.

2.4 Immunohistochemistry and cell viability stain

2.4.1 PGP 9.5 immunolabelling

PGP 9.5 is a specific marker of neuronal cells, used in immunolabelling techniques to visualize neurons. In the experiments performed in this work, PGP 9.5 immunolabelling was employed to allow clear distinction between neurons and glial cells during cell counting. PGP 9.5 immunolabelling was performed as follows: cell cultures grown for the designed period of time were washed with phosphate buffered saline (PBS, pH 7.3) and fixed with 4% paraformaldehyde for 1 hour at room temperature. Fixed cultures were washed with PBS and incubated with primary rabbit anti PGP 9.5 antibody (1:30000 in antibody diluting solution (ABDS: PBS, lysine, 0.1% sodium azide, 0.1% BSA, 1% Triton X-100) for 2 hours at room temperature, washed with PBS and incubated with secondary goat anti-rabbit antibody conjugated with fluorescein (1:100 in ABDS) for one hour at room temperature. After incubation cultures were washed with PBS and mounted on glass slides in Citifluor mountant.

2.4.2 Bis-benzimide/propidium iodide staining

4 hours after exposure to H_2O_2 the culture medium was removed and replaced with 1ml of fresh medium. 10µl of bis-benzimide (Hoechst stain) (stock 500µg/ml in PBS) was added to each well. After 20 minutes incubation at 37°C 10µl of propidium iodide (PI) was added and cultures were incubated at room temperature for 5 minutes. Subsequently all wells were

washed two times with HBSS without phenol red (Sigma), fixed for1 hour in 4% glutaraldehyde, washed twice in PBS and mounted on glass slides in Citifluor mountant.

2.4.3 Catalase/SOD Cu/Zn immunolabelling

Cultures fixed as described above were blocked for one hour with mixed 5% donkey and rabbit sera in PBS, and subsequently incubated with polyclonal rabbit anti-catalase antibody (diluted 1:500 in ABDS) for 6 hours at room temperature. After incubation cells were washed with PBS and incubated overnight at 4°C with sheep polyclonal antibody against superoxide dismutase Cu/Zn (diluted 1:500 in ABDS). Cultures with bound primary antibodies were washed again with PBS, and incubated for 1 hour at room temperature with the donkey anti rabbit secondary antibody conjugated with fluorescein (1:100 in ABDS) washed with PBS and incubated with polyclonal anti sheep secondary antibody conjugated with rhodamine red for two hours at room temperature (diluted 1:100 in ABDS). Stained cultures were mounted on glass slides in Citifluor and viewed under X400 magnification using Zeiss Axiophot microscope. Specificity of secondary antibodies was confirmed by immunolabelling of cultures with omission of primary antibodies; both secondary antibodies failed to immunolabel the cultures without pretreatment with respective primary antibodies.

2.5 Quantification of cell numbers

Cells stained with PGP 9.5 or bis-benzimide and propidium iodide were counted under 400 X magnification using either Zeiss Axiophot or Nikon Eclipse EB800 microscope. Bis-

benzimide and propidium iodide stained cells were counted in five random fields of view on each coverslip, while PGP 9.5 stained cells were counted in a strip across the diameter of the coverslip. Data were obtained from three experiments and pooled for subsequent statistical analysis.

2.6 PI-3K inhibition

2.6.1 Preparation of cell cultures

Cell cultures prepared as described above were allowed to grow for 24 hours after seeding. NT-3 and GDNF were then added to the cultures at 10ng/ml concentration. Where appropriate, the specific inhibitor of PI-3 kinase, LY 294002 at constant concentration of 300 μM was added to the cells 30 minutes prior to factor treatment. Subsequently cells were grown for 12 or 36 hours at 37°C, 2.5 % CO_2, fixed in 4% paraformaldehyde for 1 hours at room temperature and processed for PGP 9.5 immunostaining or stored at +4°C for further use, storage time did not exceed 7 days.

2.6.2 Preparation of cell lysates

For obtaining cell lysates, cultures of enteric ganglion cells were grown for 24 hours after seeding, and where appropriate treated with LY 294002 inhibitor at a concentration of 300μM concentration. 30 minutes later cells received NT-3 at 10ng/ml concentration. Cells were collected on ice 5, 20, 35 and 55 minutes after NT-3 treatment, washed three times with cold

PBS and scraped into NP40 lysis buffer (PBS, 1%NP40 detergent, NaCl, Tris pH 8.0, protease inhibitor cocktail (Boeringer). Subsequently cells were incubated on ice for 30 minutes and spun at 10000 rpm, 4°C for 10 minutes and supernatants were collected and stored at -20°C for protein quantification. Average protein yield per one condition was 0.7mg/ml.

2.7 Western blot analysis

After 12 or 36 hours incubation with factors (NT-3 or GDNF at 10ng/ml concentration) protein lysates from the cultures were prepared in the same way as described above and stored at -20°C. Protein assay was performed using Bio Rad protein assay kit, according to manufacturer's protocol. Standard curves were prepared for each session of protein determination from known quantities of bovine serum albumin (Sigma).

Protein lysates obtained from the cultures were processed as follows.
Desired amount of samples was loaded onto 15% SDS-PAGE gels. Proteins were separated in electrode buffer (25mM Tris, 192mM glycine, 0.1% SDS) at 120V for
3 hours and transferred to nitrocellulose membranes by electroblotting in transfer buffer (25mM Tris, 192mM glycine, 0.1% SDS, 20% methanol) at 100V for one hour. Equal protein loading was checked after electroblotting by incubating membranes containing transferred proteins with ponceau red (Sigma), which allowed visual detection of the proteins present on the membranes.

To obtain an estimate of the amount of catalase and SOD Cu/Zn detected on the membranes, and also to check the sensitivity of assay, standard curves were prepared using from 50 to 800ng of purified catalase or SOD Cu/Zn protein (Sigma).

Membranes were blocked in PLT (PBS, 5% non fat dry milk, 0.1% Tween 20) overnight at 4°C, and processed for immunoblotting as described above.

Briefly, blots were incubated with primary antibodies (see table 2.1), diluted in PLT for two hours at room temperature, washed three times, 10 minutes each time with PLT and incubated with secondary antibodies conjugated with horseradish peroxidase for one hour at room temperature with two subsequent washes in PBS containing 1% Tween 20 and a final 10 minutes wash with PBS. Blots were developed using chemiluminescence kit (Amersham). Exposed films were processed on an automated Kodak X-OMAT processor and digitised using alpha imager software; optical density was measured using Scion Image software (Scion Corporation).

Densitometry was used in order examine the differences between bands obtained in western blots. Briefly, a diagonal strip across the image of western blot membrane, containing the bands was selected. Subsequently, software package measured intensity of bands and prepared a densitometric graph with peaks which area depends on the intensity of bands. Optical density (OD) values, corresponding to the areas under peaks were then gathered from several western blot membranes and used to calculate mean OD value of a band obtained for a given protein.

Before reprobing, each membrane was incubated in stripping buffer (0.1M glycine, 0.1M Tris-HCl, pH 2.0) for 15 minutes at room temperature, washed in neutralizing buffer (Tris-HCl pH 8.8) and blocked in PLT for one hour at room temperature.

Primary and secondary antibodies used for immunoblotting	Supplier	Dilution
Anti Catalase	Calbiochem	1:2000
Anti SOD Cu\Zn	Calbiochem	1:2000
Anti bcl 2	Oncogene	1:1000
Anti c-fos	Oncogene	1:1000
Anti ERK2	Calbiochem	1:1000
Anti phospho ERK	Calbiochem	1:2500
Anti phospho MEP 1/2	Sigma	1:1000
Anti phospho Akt	Santa Cruz	1:500
Secondary antibodies (conjugated with horseradish peroxidase)		
Donkey anti sheep	Sigma	1:5000
Goat anti rabbit	Pierce	1:5000

Table 2.1 Antibodies and their concentrations used in this work.

2.8 Statistical analysis

Data obtained in the experiments was analysed by one-way analysis of variance (ANOVA), at $p < 0.05$ using Origin software package.

Chapter 3

The effects of neurotrophic factors on cultured enteric ganglion cells subjected to H_2O_2 induced oxidative stress

Summary

The experiments described in this chapter were undertaken in order to elucidate the effects of NT-3 and GDNF on the survival of enteric ganglion cells exposed to oxidative stress *in vitro*. Hydrogen peroxide (H_2O_2) exposure is a well-established way of inducing oxidative stress that has been used in a variety of experimental models [Fuson et al. 1999, Mishel et al. 1997] therefore it was chosen as the means of inducing oxidative stress in these experiments. To test the potential of H_2O_2 to induce cell death in enteric ganglion cells, cultured enteric ganglia were exposed to a range of H_2O_2 concentrations for 6 hours, followed by measurement of cell viability using MTS assay. As expected, H_2O_2 decreased cell viability in a dose dependent manner.

Next, effects of NT-3 and GDNF on the viability of cells exposed to H_2O_2 were assessed. NT-3 showed a slight protective effect which, although only bordering on significance was observed at every concentration of H_2O_2. In contrast, GDNF did not increase the viability of cells exposed to oxidative stress. In order to investigate the possible differential effect of H_2O_2 treatment on the neuronal and glial populations, cultures of enteric ganglia were exposed to H_2O_2 and subsequently immunolabelled with a pan neuronal marker, PGP 9.5. Cell counts confirmed the results of MTS assay, showing a decline in both neuronal and glial cell numbers with increasing concentrations of H_2O_2, also indicating that neurons are more vulnerable to oxidative stress than glial cells.

In order to study the effects of NT-3 and GDNF on H_2O_2 treated cultures more closely, cultures exposed to H_2O_2 for 4 hours were stained with Hoechst and propidium iodide dyes, allowing distinction between live and dead cells. Cell counts performed on stained cultures

confirmed the MTS data. NT-3 decreased the number of dead cells present in both H_2O_2 treated and control cultures. GDNF did not have a significant effect on the numbers of propidium iodide stained cells. Instead, it increased total number of cells in the control cultures. However, H_2O_2 treatment abolished that effect. Interestingly, cell viability in cultures that received mixed NT-3 and GDNF treatment was similar to that in cultures that did not receive factor treatment. These data suggests that NT-3 might play a role in protecting enteric ganglion cells from oxidative stress *in vivo*.

3. Introduction

Is now well established that neurotrophic factors are crucial for survival of neuronal populations during development [Yamamori et al. 1992] and for the maintenance of existing neuronal circuitry. Recent evidence, however, shows that neurons are also dependent on neurotrophic factor support in another context, that is when they face adverse conditions [Heaton et al. 2000, Miyazaki et al. 1999], including oxidative stress. While low levels of free radicals are important in cell to cell interactions [Lipton et al. 1997, McCord et al. 1995], it is known that high levels of free radicals are damaging. Oxidative stress has been implicated to be the cause of neuronal loss in many disorders, and also in aging [Harman et al. 1992]. Supporting this idea is the fact that increased oxidative damage has been demonstrated in many human neurodegenerative disorders [Coyle et al. 1993]. There is evidence demonstrating improved neuronal survival after neurotrophic factor treatment during pathological conditions, such as Parkinson's disease [Zurn et al. 2001] although the molecular mechanisms underlying this effect are still not completely understood.

Most research so far has concentrated on the CNS. However, there are data indicating that free radical damage may also play a role in the decrease of the neuronal numbers observed in the ENS during aging [Cowen et al. 2000]. As in the case of the CNS, there is some evidence suggesting that neurotrophic factors can protect enteric neurons from oxidative damage. For example, it has been shown that NT-3 and GDNF treatment lowers free radical production in the rat gut *in vitro* [Thrasivolou et al. 2000]. Despite the fact that our understanding of both signalling pathways and protective mechanisms activated by neurotrophic factors still requires more research, it is likely that neurotrophic factors would employ similar mechanisms to protect neuronal populations of the CNS and ENS. If that is the case, then data describing protective effects of neurotrophic factors in the CNS gathered so far could give valuable clues to understanding the effects of neurotrophic factors on ENS neurons.

3.1 Effects of oxidative stress on neuronal populations of the CNS and ENS

As described in the general introduction, (section 1.5), neurons are exposed to attack by free radicals throughout their life, resulting in a whole range of deletorious effects.
When exposed to oxidative stress, neurons can die either by necrosis or apoptosis. Apoptosis is also called programmed cell death, and involves activation of a cascade of proteins participating in the process, as well as synthesis of new proteins required for progression of the process. The characteristic morphological features of apoptosis include shrunken nuclei with condensed chromatin and autophagic vacuoles in the cytoplasm, as opposed to necrotic cell death which involves swelling of the cellular membrane and intracellular organelles, eventually leading to spilling of the cellular contents into the surrounding tissue [Banasiak et

al. 2000]. Most often dying cells do not display characteristics of only one of the two modes of death, but rather show morphologies including traits of both apoptosis and necrosis.

Among many other stimuli, incubation of cultured PC 12 cells in a high oxygen atmosphere results in their apoptotic cell death, mediated by ROS [Kubo et al. 1996]. Substances that increase the ROS content in cells, such as hydrogen peroxide, singlet oxygen and peroxynitrate all cause death of neurons *in vitro* and *in vivo* by apoptosis or necrosis, depending of the severity of the oxidative stress [Hoyt et al. 1997, Ming et al. 2001, Regan et al. 2001, Liang et al. 2000]. Neuronal death may also follow downregulation of cellular antioxidant systems. After inhibition of cysteine uptake and consequent decrease of glutathione synthesis or after exposure to high extracellular levels of glutamate, cultured neurons die through a mechanism intermediate between apoptosis and necrosis [Tan et al. 1998]. Downregulation of superoxide dismutase (SOD) in embryonic spinal neurons and PC12 cells has a similar effect, causing enhancement of the production of ROS and induction of apoptosis [Troy et al. 1996].

As mentioned previously, oxidative stress has been implicated in a variety of very different human neurodegenerative disorders. Although the specific sources of the free radicals and the neuronal populations affected differ between the particular disorders, there are many similarities in their effects. Increased levels of oxidatively altered proteins, lipids and nucleic acids, accompanied by increased levels of antioxidant enzymes and other stress response proteins has been detected in post mortem tissue samples from Parkinson's disease patients [Dexter et al. 1986]. Disturbances of mitochondrial metabolism are also common, which might contribute to the increased levels of oxidative damage through the leakage of ROS from the mitochondrial respiratory chain in Alzheimer's disease [Behl et al. 2002]. These

observations implicate oxidative stress as a main cause of pathogenesis of such disorders as Alzheimer's disease, and are backed up by both histopathological and experimental evidence. It is known that increased levels of cellular components damaged by ROS appear early compared to other pathological changes in these disorders [Pratico et al. 2001]. Post mortem analyses of brains from Parkinson's patients have shown an increase of both ferric and total iron in the affected brain regions, which could contribute to the production of hydroxyl anion from hydrogen peroxide [Dexter et al. 1989]. Aggregates of amyloid β (Aβ), found in the senile plaques as the major symptom of Alzheimer's disease were proved to indirectly and directly induce oxidative stress in cells *in vitro* and *in vivo* [Behl et al. 1999 and 1994, Mark et al. 1997, Keller et al. 1997]. By impairing the function of membrane ion transporters and promoting membrane depolarisation Aβ induced lipid peroxidation renders neurons vulnerable to apoptosis. These processes are thought to be involved in increasing the amount of free radicals present in the cells leading to induction of oxidative stress. This view is supported by studies showing that antioxidants, such as vitamin E, uric acid, propyl gallate, glutathione and estrogens can protect neurons from Aβ toxicity [Stull et al. 2002].

Not much is known about the involvement of oxidative stress in the pathologies of the PNS, including the ENS, or the changes observed in the ENS during aging. There is some evidence however, suggesting that oxidative stress has similar, deletorious effects on the neuronal populations of both the ENS and CNS. It has been demonstrated that pretreatment of intestinal segments with hydrogen peroxide or cumene hydroperoxide resulted in a damaged contractile response of the longitudinal smooth muscle to methacholine in all parts of the GI tract, and diminished maximal response as well as decreased contractions [Van der Vilet et al. 1989]. Moreover, due to their location, it can be argued that enteric neurons are at greater risk of oxidative injury than neurons of CNS. Ingested food contains substances that can increase free

radical formation, for example a diet containing excess iron might cause raise in the levels of free radicals [Halliwell et al. 1986] especially in the situation where the intestine was iron deficient before receiving iron rich diet [Srigirdhar et al. 1998]. Taken together, it is feasible to assume that oxidative stress might play a role at least in some ENS disorders, as well as aging.

3.2 Survival promoting effects of neurotrophic factors

As described above, neurotrophic factors are known to promote the survival of neurons at different developmental stages, as well as play a role in maintenance and stress resistance of adult neuronal cells. A considerable amount of evidence has accumulated over the years to support this view, using both *in vivo* and *in vitro* models. Protective effects of neurotrophic factors on neuronal populations extend beyond developmental changes. Data collected by many laboratories supports the view that, in addition to participating in the normal development of the nervous system, neurotrophic factors also protect neurons facing adverse conditions, such as ischemia or various chemical insults. NGF has been implicated in protecting cerebellar granule cells from ethanol toxicity *in vitro*, possibly by activating the PI3 kinase signalling pathway [Heaton et al. 2000], while GDNF has been shown to protect rat hippocampal neurons *in vivo* from death after transient ischemia [Miyazaki et al. 1999], as well as dopaminergic neurons from 6-hydroxydopamine (6-OHDA) neurotoxicity [Yurek et al. 1999].

Many of the conditions causing neuronal death either directly or indirectly affect the redox status of the cells, increasing the cellular concentration of free radicals [Hoyt et al. 1997]. Considering this fact, it seems likely that neurotrophic factors might protect neurons at least in

part by increasing their ability to cope with oxidative stress. One possible mechanism by which trophic factors increase cellular resistance to free radicals could be by enhancing neuronal antioxidant defences [Mattson et al. 1996] and inhibiting neuronal death induced by oxidative stress, which has been documented *in vitro* using cultured neurons [Klocker et al. 1998]. NGF has been demonstrated to protect aged CNS *in vivo* [Nistico et al. 1992] and PC12 cells *in vitro* [Sampath et al. 1994] against free radical damage, possibly by the means of diminishing of the intraneuronal free radical concentration [Dougan et al. 1997], although its neuroprotective effects do not always employ this mechanism [Satoh et al. 1996]. In addition to the possible inhibition of direct oxidative attack by free radicals, neurotrophins may also act directly through redox sensitive transcription factors, influencing glutathione redox status, and enhancing the expression of antioxidant enzymes in the cell cultures [Mattson et al. 1997].

GDNF has been reported to have antioxidant properties similar to these of neurotrophins. When administered into striatum of adult rats, it simultaneously increased the levels of many antioxidant enzymes involved in the metabolism of free radicals, which suggests that GDNF can maintain balance between antioxidant enzymes in the cell, as well as facilitate efficient removal of free radicals [Chao et al. 1999]. GDNF has also been shown to protect mesencephalic neurons *in vitro* from apoptosis induced by oxidative stress, by activating PI3 kinase and its downstream target, Akt [Sawada et al. 2000].

3.3 Aims

Neurotrophic factors are known to protect various neuronal populations from cell death induced by oxidative stress. However, most research has concentrated on the CNS; there are relatively few reports concerning the actions of neurotrophic factors on the neurons of the PNS. The aim of the work described in this chapter was to investigate the effects of NT-3 and GDNF on cultures of enteric ganglion cells exposed to oxidative stress, using an MTS cell viability assay. Cell cultures exposed to oxidative stress were also stained using propidium iodide and bis-benzimide (Hoechst stain) in order to examine the effect of oxidative stress and factor treatment on both total and dead cell numbers in the cultures. The possible differential effects of H_2O_2 exposure on neurons and glial cells were examined by counting surviving cells in the cultures immunostained with PGP 9.5.

3.4 Methods

The methods used in this chapter are described in chapter 2 (sections 2.1, 2.2, 2.3, 2.4.1, 2.4.2 and 2.5)

3.5 Results

3.5.1 Assessment of H_2O_2 toxicity on cultured enteric ganglion cells.

To determine an appropriate range of H_2O_2 concentrations for those experiments, cultures of enteric ganglion cells established as described in materials and methods (see chapter 2, section 2.1) were allowed to grow for 24 hours after seeding, and subsequently exposed for 6 hours to H_2O_2 at concentrations of 1, 5, 10 and 25μM. Results of three separate experiments are summarized in Fig. 3.1. The MTS assay data show a dose-dependent decrease in cell viability with increasing levels of H_2O_2. The highest concentration of H_2O_2 eliminated all the cells from the culture at the time of viability assay. Therefore, it was not used in further experiments.

The cultures of dissociated myenteric plexus ganglia used in this study contain both neurons and glia [Saffrey et al. 2000]. In order to investigate the possible differential effects of H_2O_2 on neuronal and glial populations present in the cultures, cells exposed to H_2O_2 for 6 hours were subsequently fixed using paraformaldehyde and immunolabelled with antibody against the general neuronal marker PGP 9.5.

Cells in the immunostained cultures were counted as described in materials and methods (chapter 2, section 2.5). A summary of the data for cell counts is shown in Table 3.1. Both neuronal and glial populations were found to diminish with increasing concentrations of H_2O_2, confirming the results of the MTS assays.

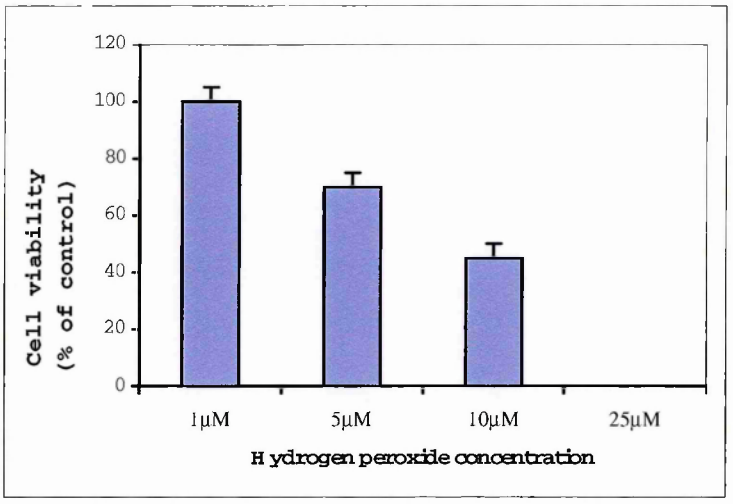


Figure 3.1 The effect of different concentrations of H_2O_2 on viability of cultured enteric ganglion cells. Results are expressed as the percentage of untreated controls. Data are presented as a mean of 3 separate experiments, each treatment done in 4 replicas in each experiment. Error bars represent ± S.E.M.

Treatment	Neurons	± SE	Glia	± SE	Neuron/Glia ratio
Control	440	9.3	912	14.6	0.48
5 μM H_2O_2	129	13.9	404	33.6	0.32
10 μM H_2O_2	15	1	88	5.1	0.17
25 μM H_2O_2	1	0.3	15	2.6	0.06

Table 3.1 The effect of increasing concentrations of H_2O_2 on enteric neurons and glial cells *in vitro*. Cell cultures were exposed to H_2O_2, fixed in 4% paraformaldehyde and subsequently stained with an antiserum to PGP 9.5. Cells were counted in a strip across the diameter of the coverslip, under X400 magnification using a Zeiss Axiophot microscope. Data in the table represents mean of cell numbers collected in 3 separate experiments, with at least 3 replicas for each condition per experiment.

However, neurons seemed to be more vulnerable to oxidative damage than glia, as indicated by greater rate of decline of neuronal numbers compared to glia with increasing concentrations of H_2O_2. This trend is also indicated by analysis of neuronal to glial cell ratio, showing the shift towards the glial component with increasing concentrations of H_2O_2.

3.5.2 Effects of NT-3 and GDNF on the viability of enteric ganglion cells exposed to oxidative stress.

To answer the question of whether NT-3 and GDNF can protect enteric ganglion cells from the deleterious effects of oxidative stress, cell cultures were supplemented with 10ng/ml of NT-3 and GDNF 12 hours prior to exposure to H_2O_2. This time point was chosen to reduce the effects of the known trophic actions of these factors on the cultures [153]. Once again, the MTS cell viability assay was used as a mean of measuring cell viability. Figure 3.2 illustrates the effect of incubating cell cultures with NT-3 for 12 hours before exposure to H_2O_2.

NT-3 treated cultures displayed an increase in cell viability at every tested concentration of hydrogen peroxide, suggesting that NT-3 may be able to able to partially protect enteric ganglion cells exposed to oxidative stress. Statistical analysis of the data shows that although the difference between NT-3 treated cells and controls is consistent, it is only bordering on significance ($p<0.1$). Similar experiments performed using GDNF

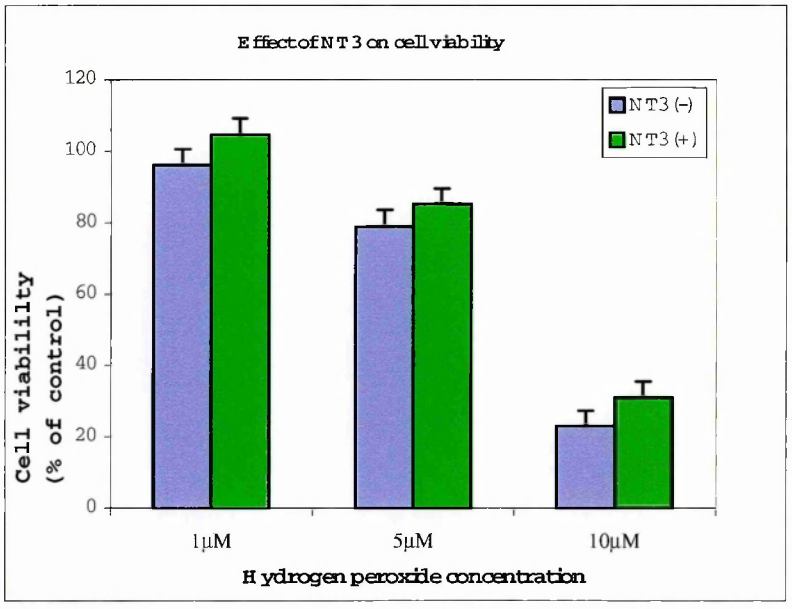


Figure 3.2 Viability of cells treated with NT-3 (10ng/ml) prior to H_2O_2 exposure, compared with untreated controls. Values represent mean absorbance for each treatment obtained from three separate experiments, expressed as a percentage of untreated controls. Data are presented as a mean of 3 separate experiments, each treatment in four replicas per experiment. Error bars represent ± S.E.M.

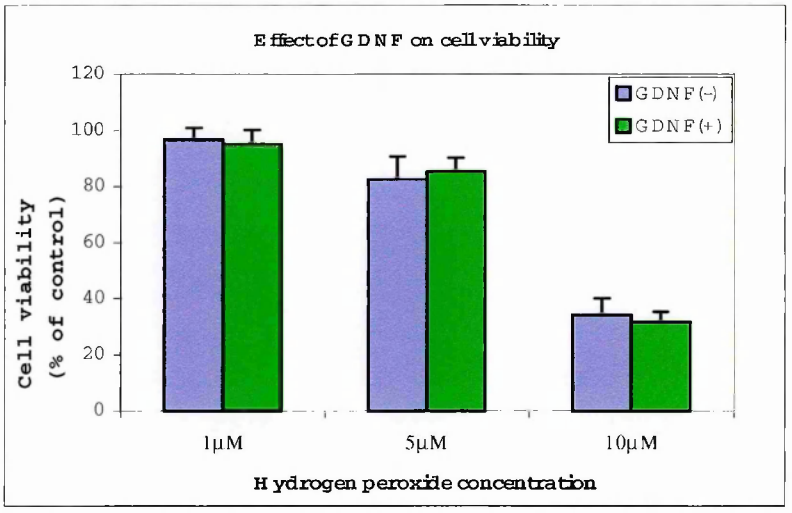


Figure 3.3 Viability of cells treated with GDNF (10ng/ml) prior to H_2O_2 exposure, compared with untreated controls. Values represent mean absorbance for each treatment obtained from three separate experiments, expressed as a percentage of untreated control. Data are presented as a mean of 3 separate experiments, each treatment in four replicas per experiment. Error bars represent ± S.E.M.

showed no change in cell viability between controls and factor treated cells, indicating that GDNF is unable to rescue enteric ganglion cells exposed to oxidative stress (Fig. 3.3).

3.5.3 Hoechst/Propidium Iodide staining of cell cultures exposed to H_2O_2 combined with NT-3 and GDNF treatment

To complement the results obtained in the previous experiments, cells incubated with NT-3, GDNF or both, and subsequently exposed to oxidative stress were fixed and stained with bis-benzimide and propidium iodide (also known as live/dead stain) after 4 hours incubation with H_2O_2. Both dyes stain cell nuclei, binding to the nucleic acids. While bis-benzimide is retained in the cells regardless of their status, propidium iodide is excluded from living cells, thus providing a way of measuring both total cell numbers and the extent of cell death. In this set of experiments only a single concentration of H_2O_2 was chosen based on MTS assay data, which showed roughly 40% cells surviving at 10μM. NT-3 and GDNF were used as in previous experiments at 10ng/ml. The effect of combined NT-3/GDNF treatment was also investigated using 10ng/ml of each factor. Cells stained with bis-benzimide and propidium iodide (Fig. 3.4) were subsequently counted, and analysed using one way ANOVA at $p<0.05$.

As expected, total (stained with Hoechst dye) cell numbers decreased in the control cultures exposed to H_2O_2 induced oxidative stress, compared with the untreated cultures ($p<0.02$, Table. 3.2).

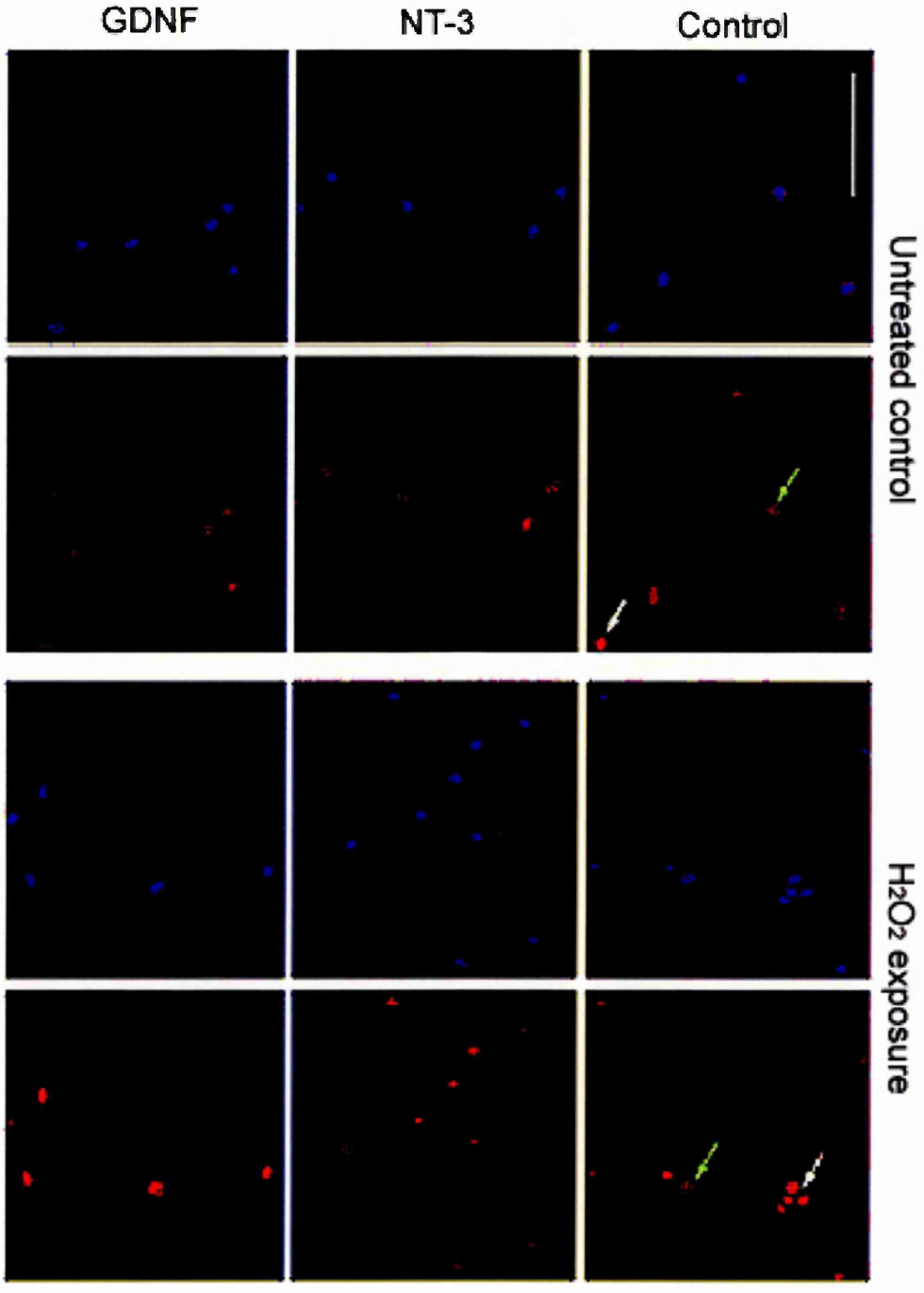


Fig. 3.4 Hoechst (blue) and propidium iodide (red) staining of cultures exposed to H_2O_2 and controls, grown with NT-3 or GDNF (10ng/ml). Cultures were stained as described in materials and methods (see chapter 2, section 2.4.2). White arrows show strongly stained cells, indicative of cell death as opposed to background staining of live cells (green arrows). Pictures were taken at X400 magnification using a Zeiss Axiophot microscope. White bar at top right represents 50μm

Treatment	(-)H_2O_2 Hoechst	PI*	(+)H_2O_2 Hoechst	PI*
Control	453	155	425	184
NT-3	434	144	431	141
GDNF	509	158	427	161
NT-3/GDNF	456	155	405	168

Table 3.2 Total cell numbers counted in the cultures grown for 12 hours with NT-3, GDNF or both (each factor at 10ng/ml concentration), treated and untreated with H_2O_2. Cells were counted in 5 random fields of view across the coverslip under X 400 magnification, using a Zeiss Axiophot microscope. Data represent the mean of the cell numbers gathered in three separate experiments. * Propidium iodide

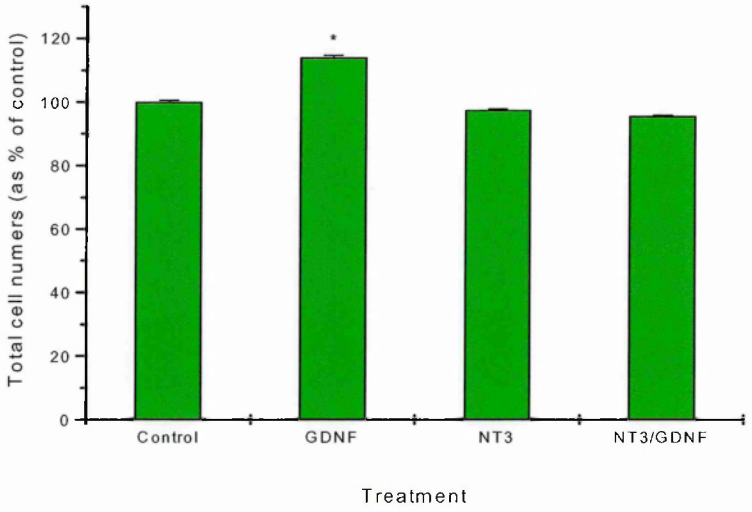


Figure 3.5. The effect of NT-3, GDNF and combined factor treatment on total (stained with bis-benzimide) cell numbers in the cultures of enteric ganglion cells. Factors (10ng/ml concentration) were added 12 hours prior to beginning of the experiment. Data are presented as a mean of 3 separate experiments. Error bars represent ± S.E.M. Asterisk marks statistically significant difference.

GDNF increased total cell numbers present in the non-stressed cultures compared to controls ($p<0.04$, Fig 3.5), although this effect was abolished when the cultures were exposed to H_2O_2. In agreement with the data obtained with MTS assay (Fig. 3.3), GDNF treatment was not able to rescue cells in H_2O_2 treated cultures (Fig. 3.6)

In contrast to GDNF, NT-3 did not have any effect on total cell numbers, instead significantly decreasing the number of dead (stained with propidium iodide) cells in the cultures exposed to hydrogen peroxide compared with H_2O_2 treated controls ($p<0.002$, Fig. 3.6, Table 3.2). An interesting observation was that combined NT-3/GDNF treatment seemed to abrogate both the proliferative effect of GDNF as well as protective effect of NT-3 (Fig. 3.5 and 3.6)

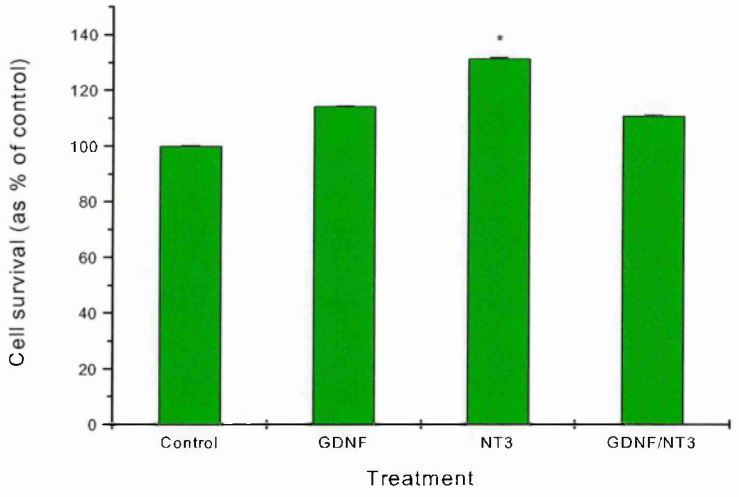


Figure 3. 6 The effect of NT-3, GDNF and combined factor treatment on cell survival. Presented values were calculated as a ratio of total cell numbers to the number of dead cells and expressed as a percentage of control. Factors (10ng/ml) were added 12 hours prior to H_2O_2 treatment. All conditions received 10μM of H_2O_2 and were stained with bis-benzimide and propidium iodide 4 hours after exposure to H_2O_2. Data are presented as a mean of 3 separate experiments. Error bars represent ± S.E.M. Asterisk marks statistically significant difference.

3.6 Discussion

The experiments described in this chapter were conducted in order to investigate whether NT-3 and GDNF can protect enteric ganglion cells exposed to oxidative stress. H_2O_2 was chosen to induce oxidative stress for several reasons. (i) H_2O_2 is one of the established models of inducing oxidative stress *in vitro* [Fuson et al. 1999, Mischel et al. 1997, Gotz et al. 1999]. (ii) Exogenous H_2O_2 can readily enter the cells due to its high membrane permeability [Sampath et al. 1994], and can induce apoptosis in many cell types [Clement et al. 1998]. (iii) It is one of the naturally occurring oxygen radicals, arising as a product cellular metabolism, and it can give raise to many highly oxidizing radicals such as hydroxyl radical; it is also responsible for oxidative damage to the cells *in vivo* [Sastry et al. 2000]

It is well documented that both NT-3 and GDNF are able to rescue broad range of neuronal populations from various adverse conditions, including oxidative stress [Thrasivolou et al. 2000, Mattson et al. 1997, Sawada et al. 2000].

NT-3, a member of the neurotrophin family, in addition to acting as a differentiation factor in the development of ENS and promoting development of neurons and glia from precursor cells [Semkova et al. 1999], also acts as a survival factor for various neuronal populations at certain stages of their development [Davies et al. 1994]. Similarly GDNF has been shown to be critical for the survival and proliferation of enteric precursor cells [Enomoto et al. 1998], as well as other classes of neurons. Both of these factors are present in the ENS throughout the life and have been shown to be necessary for survival of the enteric neurons during the development of ENS. Considering these facts, one might expect NT-3 and GDNF to serve as survival factors for enteric neurons facing adverse conditions, presuming such neurons express appropriate receptors, which has been shown for NT-3 [Sternini et al. 1995], it has been

suggested that the same might be true for GDNF, which is confirmed by the responsiveness of neurons and glial cells used in current experiments to both NT-3 and GDNF stimulation.

The data presented in this chapter show that NT-3 may protect enteric ganglion cells from cell death induced by exposure to H_2O_2, as demonstrated by MTS cell viability assay and cell counts of cultures stained with propidium iodide and Hoechst dyes. The protective activity of NT-3 observed using the MTS assay was not dramatic. However, it was consistently observed at every tested concentration of H_2O_2. The protective effects of NT-3 were much more pronounced when examined by cell counts of Hoechst/propidium iodide stained cells, showing an almost 24% reduction in the numbers of dead cells after exposure to H_2O_2, compared to control cultures. The difference in strength of protective effect of NT-3 observed using MTS assay and Hoechst/propidium iodide staining could be explained in two ways. First, MTS assay offers only general information about the status of the cells in cultures, while Hoechst/propidium iodide staining in conjunction with cell counts offers much more accurate information, which allows detection of fine changes in cell death/survival. Another possible reason for the difference in the extent of protective effect of NT-3 is the different times of H_2O_2 exposure used for MTS assay and Hoechst/propidium iodide staining. After addition of H_2O_2, cell cultures were incubated for 6 hours before commencing MTS assay; to 4 hour incubation used for Hoechst/propidium iodide staining. Although H_2O_2 undergoes rapid decomposition in the culture medium even at highest, 25μM concentration used in the experiments, vanishing in about 30 minutes, cell death induced by oxidative stress continues, which might contribute to the differences in the protective effect of NT-3 observed using MTS assay and Hoechst/propidium iodide staining.

Protective effect of NT-3 on enteric neurons observed in this work remain in keeping with the reports of Thrasivolou et. al and Mattson et. al. [Thrasivolou et al. 2000, Mattson et al. 1995], showing protective effects of the neurotrophins against free radical damage as well as decrease in generation of free radicals. In contrast, Kirschner et.al. reported a lack of protective effects of NT-3 on stratial neurons of neonatal rats after MPP treatment, which blocks mitochondrial respiratory chain [Kirschner et al. 1996]. This discrepancy might be explained in several ways. First of all, models used for assessing protective effect of trophic factors differ in both studies. Secondly, the type of neurons employed (neonatal stratial neurons versus postnatal enteric neurons) might also contribute to the observed differences. Another explanation may be that different factors were used to induce neuronal death in these studies. Here, we have used hydrogen peroxide, while 1-methyl-4-phenylpiridinium (MPP), an inhibitor of mitochondrial complex I was employed in the other study. While both induce oxidative damage; hydrogen peroxide directly, and MPP by blocking the mitochondrial respiratory chain, the type of stimuli the cell receives in each case is likely to be different, possibly contributing to the different end effects.

The data gathered in this study indicate that GDNF does not protect enteric ganglion cells from oxidative stress, instead increasing cell numbers as shown by counts of bis-benzimide stained cells. On the other hand, Sawada et. al. demonstrated that GDNF was able to rescue mesencephalic neurons *in vitro* from chemically induced oxidative stress, as well as inhibiting apoptosis [Sawada et al. 2000]. Once again, the different effects of GDNF observed by Sawada et. al. and in this study could stem from several reasons, for example different cell types and cell age, as well as the different way of inducing oxidative stress: hydrogen peroxide versus glutamate and bleomycin sulphate. Also the intracellular signalling pathways avaible

for GDNF in embryonic mesencephalic neurons and postnatal enteric neurons could be different, contributing to the different end effects observed.

One novel observation made in the present study is that combined NT-3/GDNF treatment seemed to abolish the effects of both factors, as measured by cell counts of bis-benzimide and propidium iodide stained cells. One possible explanation of this observation is the type of effect NT-3 and GDNF have on enteric ganglia. NT-3 enhances cell survival, and is also known to induce differentiation of the enteric neurons [Saffrey et al. 2000]. On the other hand GDNF induced an increase in cell numbers, possibly by promoting proliferation of the cells present in the cultures, which indicates that at the time of experiments enteric ganglion cells contain either precursor cells, or cells committed to particular phenotype but still able to proliferate. These two types of effects: growth and differentiation are mutually exclusive, hence cells that received both signals at the same time might not show any, or a decreased reaction depending on the environmental and intracellular conditions.

In conclusion, the results of this chapter demonstrate that NT-3 is able to rescue enteric ganglion cells exposed to oxidative stress *in vitro*, while GDNF does not affect cell survival, instead increasing total cell numbers present in the cultures. Also, combined factors treatment with both nullified the effects of each factors alone. The possible mechanisms by which NT-3 and GDNF exert their effects on cultured enteric neurons are investigated in the subsequent chapters.

Chapter 4

Effects of neurotrophic factors on the expression of antioxidant enzymes in the ENS

Summary

The experiments described in this chapter were undertaken to examine the effects of two neurotrophic factors, NT-3 and GDNF on the expression of antioxidant enzymes in cultures of enteric ganglion cells *in vitro*. Western blot analysis was used to assess the levels of catalase and superoxide dismutase Cu/Zn (SOD Cu/Zn) present in enteric ganglion cells after 12 and 36 hours incubation with NT-3 or GDNF. The results showed no change in levels of either enzyme at 12 hours in cultures incubated with NT-3, and a slight increase of SOD Cu/Zn levels in cultures treated with GDNF as compared to control cultures grown with no factors. At 36 hours, the levels of catalase in cultures grown with NT-3 were comparable with controls, while levels of SOD Cu/Zn decreased. In contrast, cells that received GDNF for 36 hours showed increased levels of both catalase and superoxide dismutase. The data suggest that these effects were caused by an increase in the levels of these enzymes present in the cells resulting from prolonged GDNF treatment. This observation implies that although NT-3 does not affect the levels of catalase of SOD Cu/Zn in cultures of enteric ganglion cells, GDNF is able to do so.

4.1 Introduction

As discussed in previous chapters, neurotrophic factors increase the resistance of multiple neuronal populations to adverse conditions, both during the development of neural networks as well as during adulthood. One particular type of insult causing cellular damage and decline of neuronal numbers during aging and pathological conditions in both the CNS and PNS is oxidative stress. There is evidence showing that neurotrophins can protect against oxidative

stress by increasing the expression of the enzymes responsible for conversion of free radicals into less harmful compounds, both at the RNA and protein level [Sampath et al. 1994]. Such an effect, based on an increase in the levels of antioxidant enzymes has been suggested to be the mechanism by which NGF can protect cultured hippocampal neurons against direct oxidative damage [Cheng et al. 1991]. A similar protective effect has also been observed *in vivo*, when NGF administered intraventricularly protected hippocampal neurons against ischemic damage in rats [Shigeno et al. 1991]. Another member of the neurotrophin family, BDNF, has been shown to protect dopaminergic neurons *in vivo* against MPTP, which increases intracellular concentration of free radicals [Spina et al. 1992]. There are data showing that not only NGF and BDNF, but also growth factors from other families can protect various neuronal populations from oxidative stress, by means of increasing antioxidant defences of the cells. For example it has been demonstrated that GDNF is able to protect dopaminergic neurons *in vivo* from MPP^+ toxicity by increasing activities of antioxidant enzymes [Chao et al. 1999].

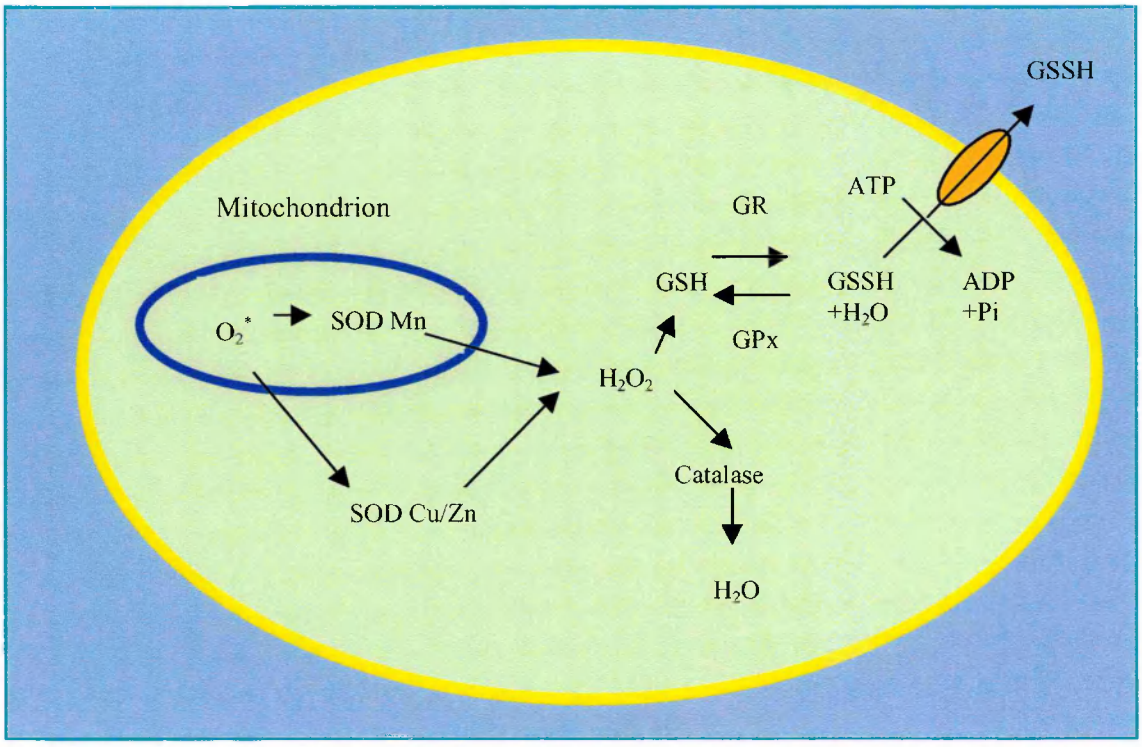
The ability of neurotrophic factors to induce increases in the levels of antioxidant enzymes is of particular interest from a clinical point of view, as it could offer a way to counteract deleterious effects of many pathological conditions which elevate cellular levels of free radicals [Coyle et al. 1993]. It has also been shown that many age-related changes affecting neurons, such as lowered ATP production, increased leakage of free radicals from mitochondria and changes in antioxidant enzymes, are directly or indirectly related to the oxidative stress neurons are exposed to during their life [Artur et al. 1992]. In this context, increased resistance to oxidative stress offered by neurotrophic factors could be employed to counteract decreases in neuronal populations of both the CNS and ENS, which is likely to be, among other things, a cause of malfunction of gastrointestinal tract often seen in elderly.

To date, caloric restriction is the only practical way of slowing down the onset of age-related changes, such as oxidative damage. Caloric restriction affects expression of a wide range of genes, many of which encode enzymes participating in energy metabolism [Dhahbi et al. 1999] which promotes more efficient energy usage, as well as enzymes involved in the metabolism of free radicals [Dhahbi et al. 1998]. It is possible that some of these effects are due to the actions of neurotrophic factors, since it has been demonstrated that caloric restriction is able to increase the levels of BDNF and other neurotrophic factors [Duan et al. 2001].

4.1.1 Antioxidant enzymes in the nervous system

To cope with oxidative stress, eukaryotic cells utilize several antioxidative defence systems that prevent accumulation of ROS. One such system is that of the antioxidant enzymes, which catalyses conversion of ROS to non-toxic products: superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) (Fig. 4.1).

Superoxide dismutases rapidly convert superoxide anion ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2). If not neutralized, H_2O_2 can undergo conversion to the more harmful hydroxy radical ($HO^{\bullet}$) in the presence of transition metals such as iron and copper. Catalase and glutathione peroxidase decompose H_2O_2 to water.


GSSH
Mitochondrion
ATP
GR
GSH
GSSH
+H2O
ADP
+Pi
O2*
SOD Mn
GPx
H2O2
Catalase
SOD Cu/Zn
H2O

in eukaryotes, it differs from the intracellular Cu/Zn SOD in terms of amino acid composition and molecular weight [Marklund et al. 1984]. In most mammals extracellular SOD is a tetrameric molecule with a high affinity for heparin. Most of the extracellular SOD, that is about 90% of total enzyme present in the organism, is localized in the extracellular matrix of tissues [Oury et al. 1997]. Localisation of extracellular SOD is regulated by the presence of a heparin-binding domain in the carboxyterminal region of the protein [Sandstrom et al. 1992].

Catalases are divided into three subgroups: monofunctional haem catalases (typical catalases), catalase-peroxidases and manganese catalases. Members of haem catalases have been found in almost all aerobically respiring organisms, both prokaryotes and eukaryotes and genes coding for typical catalases have been isolated and sequenced from more than 75 organisms. Most of these catalases are homotetramers, between 200-340 kDa in size with four prosthetic haem D groups. The majority of catalases contain ferric protoporphyrin IX, but in some representatives haem d is employed. The conserved core of typical catalases comprises about 390 amino acids, spanning four structural domains. The highest degree of homology has been found in the area around the amino acids required for binding haem, this region is highly conserved in all currently resolved structures of typical catalases [Zamocky et al. 1999]. Members of the catalase-peroxidase subgroup in eukaryotes have been found only in fungi. Their molecular weight varies from 120 to 340kDa and in general they are homodimers, although heterodimers have also been reported. The most characteristic feature of catalase-peroxidases is their bifunctional catalytic behaviour [Zamocky et al. 1999]. Manganese catalases are the smallest group of catalases, found in acid bacteria and thermophilic organisms. These enzymes are sometimes referred to as pseudocatalases due to the fact that they utilize manganese ions in their active sites instead of ferric haem.

The last group of enzymes involved in the removal of free radicals are glutathione peroxidase (GPx) and glutathione reductase (GR), utilizing glutathione to decompose H_2O_2. Glutathione (GSH) is a tripeptide consisting of glutamate, cysteine and glycine. The cellular levels of GSH are controlled by multiple enzyme systems such as γ-glutamyltranspeptidase, amino acid transporters, glutathione synthetase (GS), GPx and GR, but the rate limiting reaction in GSH synthesis is catalysed by γ-glutamyl cysteinyl synthetase (γ-GCS) [Wild et al. 2000]. This makes regulation of γ-GCS expression and activity critical for GSH homeostasis [Griffith et al. 1999]. The GCS holoenzyme is a heterodimer consisting of a catalytic, heavy subunit and regulatory, light subunit.

Under oxidative stress, ROS are reduced by GSH, which then forms the oxidized disulphide, GSSG. In addition to its action as a chemical antioxidant GSH also acts in the enzymatic conversion of H_2O_2, as a cofactor in GPx mediated reduction of peroxides, also resulting in the formation of GSSG. The next step involves reduction of GSSG to GSH by GR at the expense of NADPH. When the reductive capacity of a cell is insufficient, it causes a decrease of the GSH/GSSG ratio which in turn activates redox sensitive transcriptional factors [Sen et al. 1996]. To maintain the cellular redox balance, surplus GSSG is exported from the cells by ATP dependent transport proteins, which suggests that severe oxidative stress can deplete cellular GSH [Leier et al. 1996]. Glutathione peroxidases can be divided into two types: selenium dependent and independent. GPx play an important role in the defence against oxidative stress. Resistance to oxidants such as H_2O_2 and menadione has been increased as a result of overexpression of the glutathione peroxidase GPx1 [Mirault et al. 1991]. Conversely a GPx (-\-) mouse strain was found to be hypersensitive to the oxidant paraquat and cortical neurons from that strain have been demonstrated to be more susceptible to insult exerted by H_2O_2 [Haan et al. 1998].

4.1.2 Patterns of expression of antioxidant enzymes during aging in the nervous system and other tissues

Aging affects the expression of a whole range of genes, from metabolic enzymes and signal transduction proteins to antioxidant enzymes. There is some discrepancy in the data concerning the effects of aging on antioxidant defence systems. Age-dependent decreases in activities of antioxidant enzymes have been observed in several studies [Schisler et al. 1987] while no changes in expression of the same enzymes were detected by other groups [Liqun et al. 1998]. Decreases in both the activities of superoxide dismutases and amount of their mRNA as well as a decrease in catalase activity have been observed during aging in the liver of F344 rats [Rao et al. 1990b]. In the same study age-dependent decreases in SOD Cu/Zn and catalase, but not in GPx were observed in the brain, whereas levels of GPx in the intestinal mucosa and kidneys decreased. Somewhat contradictory to these results Liqun et. al. [Liqun et al. 1998] found that the activities of SOD Cu/Zn and SOD Mn in the heart and kidneys of F344 rats, and GPx activity in the kidneys were decreased during aging, but the activities of both SODs, catalase and GPx in brain were not affected by age [Liqun et al. 1998]. Different results have been obtained using the C57Bl mouse strain, which shows an age-dependent decrease in the brain levels of SOD Cu/Zn, GR, and catalase [Mo et al. 1995]. Data gathered by different groups suggests that the effect of aging on a particular antioxidant enzyme is influenced by strain of the animals, gender, and also the organ [Rikans et al. 1991, Egaas et al. 1995]. The effect of age on antioxidant status in the intestine varies from study to study. The activity of SOD Cu\Zn in the intestine of F344 rats aged 26 months was significantly lower than that of the intestine from F344 rats aged 6 and 16 months, however intestinal GPx levels and lipid peroxidation were unaffected by age [Xia et al. 1995]. On the other hand, in a different study the activity of SOD Cu/Zn and lipid peroxidation were markedly increased in

the intestine of aged mice [Cristiano et al. 1995]. Results obtained by Jang et al. [Jang et al. 2001], comparing effects of aging on activities of SOD Cu/Zn, GPx and lipid peroxidation in intestine of Wistar and F344 rats show no changes in respect to both aging and strain. However, levels of glutathione s-transferase were elevated in the intestine of aged animals [Jang et al. 2001].

To add to the complexity of the picture, some data suggest that aging might affect different parts of digestive tract and other tissues differently. Microarray analysis of gene expression patterns in the duodenum and colon of young versus old F344 rats shows increased expression of GS gene in the colon, but decreased expression in the duodenum [Lee et al. 2001b]. This change seems to be part of the general change in the metabolism rate in the colon versus duodenum of aged rats: while the metabolic rate of the duodenum decreases, the metabolic rate of colon is increased [Lee et al. 2001b]. Such results suggest that the end effect of age on the levels of antioxidant enzymes in the intestine is dependent on many factors, both environmental and specific to a given cellular background.

4.1.3 Effects of caloric restriction on the expression of antioxidant enzymes in the nervous system and other tissues

As discussed in the general introduction (see section 1.5), caloric restriction is the only practical means of slowing down the onset of deleterious changes associated with aging available to date. It has been demonstrated that rats and mice kept on a calorically restricted diet, exhibit an extended lifespan and also show relatively less oxidative damage at the organ and cellular level [Mote et al. 1991]. Part of the anti-aging effect of caloric restriction is

thought to be due more efficient utilization of available calories [Masoro et al. 2000], resulting in production of smaller quantities of free radicals [Ramsey et al. 2000], possibly by decreasing the rate at which mitochondria 'leak' free radicals [de Grey et al. 2001]. However, it is known that caloric restriction alters the expression of enzymes involved not only in metabolism [Dhahbi et al. 2001], but also genes encoding at least some neurotrophic factors [Dhahbi et al. 2001] which might contribute to the reduced oxidative damage observed in calorie restricted animals. There are some data regarding changes in the expression of antioxidant enzymes resulting from caloric restriction. However, the results from different groups show certain differences in the effects of caloric restriction on the expression of particular enzyme depending on the strain of animals used in the study. For example Mote et al. reported decreased expression of SOD Cu\Zn in the liver of C57Bl male mice, while Chipalkatti et al. found no changes in SOD Cu\Zn activity in the liver of swiss albino female mice [Mote et al. 1990, Chipalkatti et al. 1983]. Similar discrepancies are found in the data concerning catalase expression. Studies in male F344 rats showed an increase of catalase mRNA in diet-restricted animals correlating with retardation of the effects of age [Rao et al. 1990]. On the other hand, Mura et al. reports no changes in the protein levels of catalase in the liver of calorically restricted mice [Mura et al. 1996]. Similar differences in the results obtained by different groups are also evident in case of GPx and SOD Mn. One possible explanation is that the inconsistent results may stem from genotype differences, since genetic differences in the effects of food restriction have been noted in mice [Harrison et al. 1986]. These differences, in addition to different strains and gender variability of animals used by different groups highlights the fact that effects of caloric restriction on the protein and RNA levels of antioxidant enzymes is quite complex, and most likely regulated by multiple factors.

4.2 Aims

The data obtained in the previous chapter indicated that NT-3 may have a protective effect on enteric ganglion cells exposed to oxidative stress. One likely explanation of this observation is an increase in the levels of antioxidant enzymes present in cultures caused by NT-3 treatment, a hypothesis supported by data from other studies showing the ability of neurotrophic factors to protect cultured cells from oxidative stress by means of increasing activities and levels of antioxidant enzymes. In order to examine this hypothesis, possible changes in protein levels of SOD Cu/Zn and catalase after NT-3 and GDNF treatment were investigated by western blotting. The expression of these enzymes by neuronal and glial cells, as well as numbers of neurons and glia present in the cultures were examined using immunohistochemical staining of cells grown in the presence of neurotrophic factors.

4.3 Methods

The methods used in this chapter were described in chapter 2 (sections 2.4.2, 2.4.3, 2.6.2 and 2.7).

4.4 Results

4.4.1 Preparations of standard curves for catalase and SOD Cu/Zn

Western blotting allowed clear detection of protein levels of both SOD Cu/Zn and catalase in the range from 50 to 800 ng, as shown in Fig. 4.2. Following western blotting standard curves were prepared using a densitometry software package (Scion Image). Comparing densitometric values obtained from the samples with the values of standard curve allowed estimation of catalase and SOD Cu/Zn protein levels present in the cultures.

4.4.2 The effects of 12 hours treatment with NT-3 or GDNF on the levels of expression of catalase and superoxide dismutase Cu/Zn by enteric ganglion cells *in vitro*

The experiments described in the previous chapter demonstrated that NT-3, but not GDNF can protect enteric ganglion cells against cell death after exposure to H_2O_2.

The effect of NT-3 could have been due to an increase in the levels of expression of antioxidant enzymes induced by the factor. To test this hypothesis, possible changes in the levels of catalase and SOD Cu/Zn in response to neurotrophic factor treatment were assessed. Cell cultures grown as described in materials and methods (section 2.1) were lysed after 12 hours incubation with NT-3 or GDNF; cell lysates were subsequently analysed by western blotting. Optical density (OD) values of samples from NT-3 treated cultures collected from several experiments showed no significant changes in the levels of catalase after 12 hours incubation with the factor compared to untreated controls and only an insignificant drop in superoxide dismutase Cu/Zn levels (Fig. 4.3, Table 4.1).

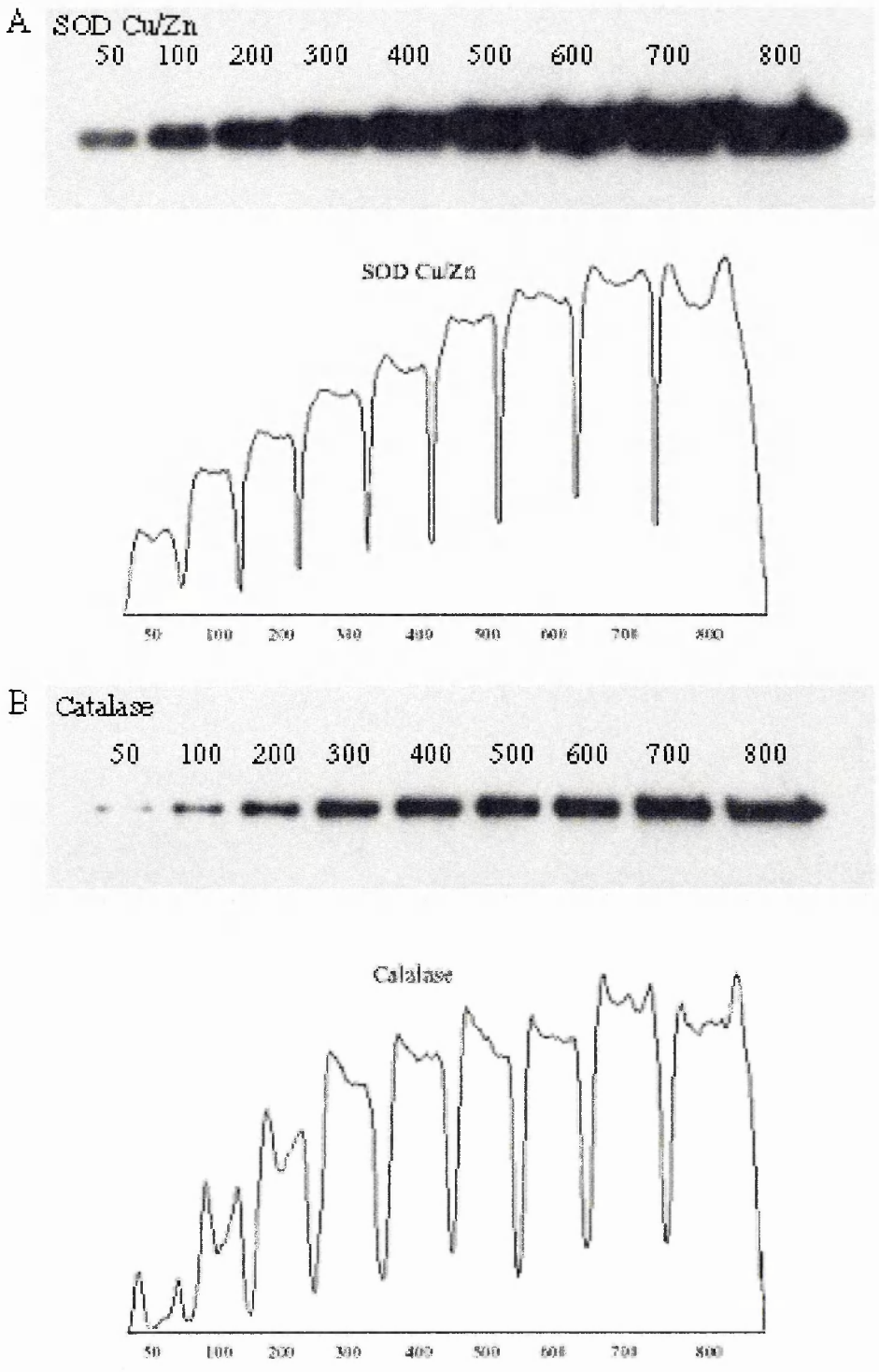


Figure 4.2 Western blots showing standard curves for SOD Cu/Zn (A) and catalase (B), and respective graphs obtained after densitometric measurement of both membranes. Values shown above the protein bands on western blots represent amounts of purified protein loaded per lane in nanograms.

Similarly, GDNF treatment had little effect on the OD values obtained from western blots stained with anti-catalase antibodies, although the decrease in the level of SOD Cu/Zn was more pronounced than in case of NT-3 treatment.

	Catalase		SOD Cu/Zn	
	12 hours	36 hours	12 hours	36 hours
NT-3	98.5 (±4.4)	96.6 (±8.2)	98.7 (±3.2)	93.3 (±2.4)
GDNF	93.6 (±9.3)	123.4 (±11.6)	91.9 (±5.7)	132 (±11.9)

Table 4.1 The effects of factor treatment on the levels of catalase and SOD Cu/Zn in the cultures grown for 12 and 36 hours. Values represent the results of densitometric analysis of western blot membranes loaded with equal amounts of protein and expressed as a percentage of respective controls. OD readings were standarized against the values obtained for total protein (ponceau red) staining of the respective membranes. Data presented in the table were gathered from at least 5 separate experiments. Values in parentheses represent ± SEM.

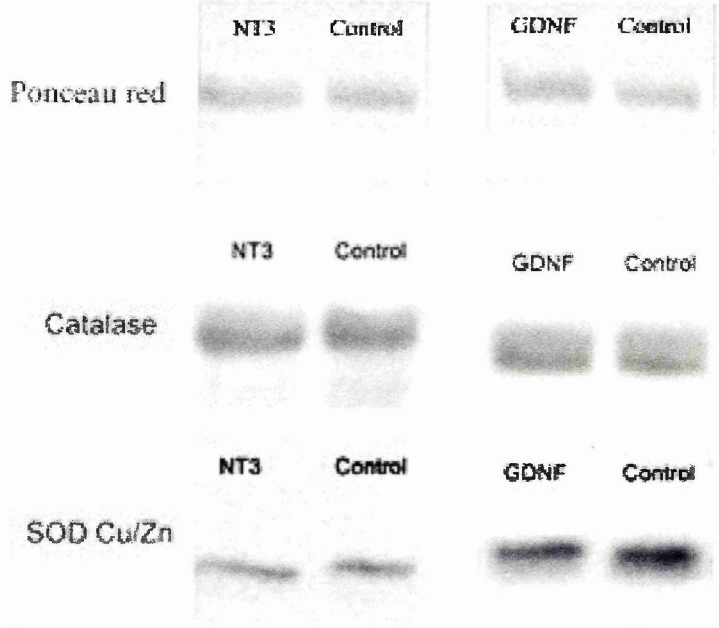


Figure 4.3 Example of western blots of lysates from sister cultures grown in the presence of NT-3 or GDNF for 12 hours prior to protein extraction, using antibodies directed against catalase (middle row) or superoxide dismutase Cu/Zn (bottom row). 5µg of protein extracts was loaded on to each gel. To ensure equal protein content on each lane after, electro blotting membranes were incubated with ponceau red (top row), and protein amounts were estimated using densitometry.

Results obtained using western blots showed little change in the levels of catalase or SOD Cu/Zn after factor treatment for 12 hours. However, the presence of both neurons and glial cells in the cultures raised the question of the relative contribution of the two cell types to the total amount of catalase and SOD Cu/Zn protein detected using western blots.

To address this issue, membranes were stained using the general neuronal marker PGP 9.5, which gives an estimate of neuronal proteins present in the lysates. Such staining would allow an estimation of the contribution of neuronal cells to the levels of enzymes detected on western blots, by comparing the amount of neuronal protein present on the membrane with the amount of catalase and SOD Cu/Zn. However, the results of preliminary experiments were not conclusive. To overcome this problem a more direct approach was undertaken: cell cultures were grown for 12 hours with NT-3 and GDNF, fixed and stained using antibodies against catalase and SOD Cu/Zn and subsequently viewed using a Zeiss Axiophot microscope. Fig. 4.4 shows the results of staining of the cell cultures using antibodies directed against catalase and superoxide dismutase. Levels of both enzymes appeared to be slightly higher in neuronal cells than in glia (white arrows point to neurons, black arrow marks glial cell). As expected, no differences in the staining intensity of the cells were observed between treatments, supporting the results obtained by western blots.

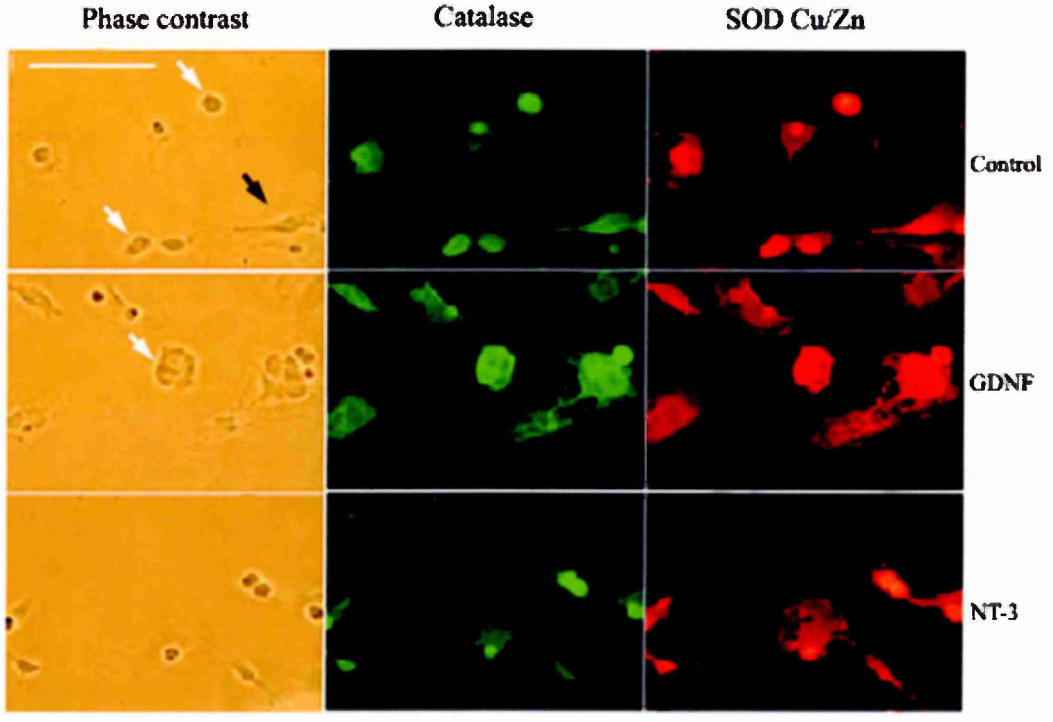


Figure 4.4 Immunohistochemical staining of cultures of enteric ganglion cells grown for 12 hours with NT-3 or GDNF. Each row shows the same field of view, pictures taken in phase contrast in the left row (yellow), middle row shows catalase immunoreactivity visualised by fluorescein (green), right row shows SOD Cu/Zn immunoreactivity visualised by rhodamine red (red). White arrows show neurons (brightly stained by both anti catalase and anti SOD Cu/Zn antibodies). The black arrow marks a glial cell. Notice the group of neurons present in the GDNF treated culture (marked with a white arrow). Pictures taken at X400 magnification using the same exposure time. White bar at the top left panel represents 50 μm.

4.4.3 The effects of 36 hours treatment with NT-3 or GDNF on the levels of expression of catalase and superoxide dismutase Cu/Zn by enteric ganglion cells *in vitro*

To examine the longer term effects NT-3 and GDNF might have on the levels of catalase and SOD Cu/Zn, cultures of enteric ganglion cells were grown in the presence of either NT-3 or GDNF for 36 hours and subsequently used for protein extraction.

Western blot analysis revealed a slight decrease in the levels of SOD Cu/Zn in the cultures treated with NT-3 compared to the untreated control cultures; levels of catalase were not significantly affected (Fig. 4.5).

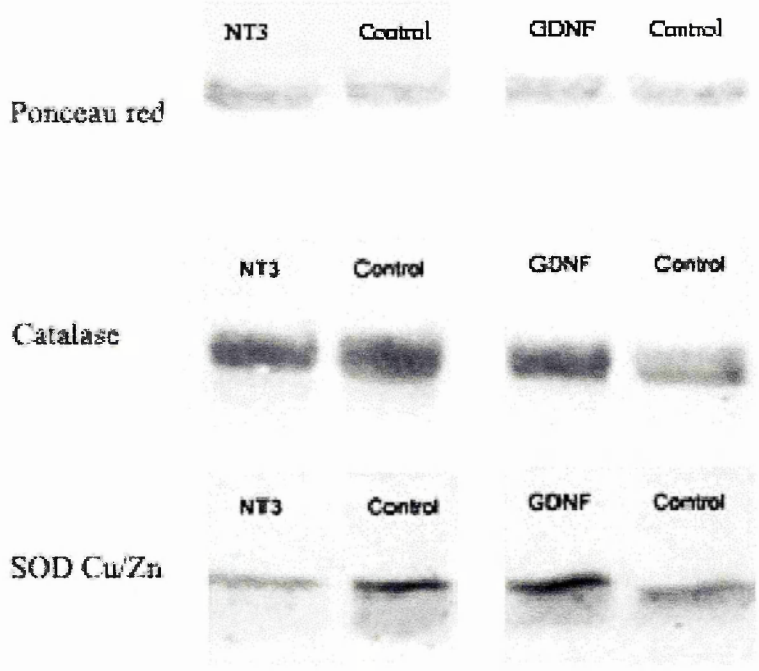


Figure 4.5 Example of western blots of lysates from sister cultures grown in the presence of NT-3 or GDNF for 36 hours prior to protein extraction, using antibodies directed against catalase (middle row) or SOD Cu/Zn (bottom row). NT-3 treatment does not change catalase levels in the cultures, instead causing a drop in the levels of superoxide dismutase, compared to untreated control cultures. GDNF treatment increased the levels of both enzymes tested after 36 hours incubation, especially SOD Cu/Zn. 5μg of protein extracts was loaded on to each gel. To ensure equal protein content on each lane, after electro blotting membranes were incubated with ponceau red (top row), and protein amounts were estimated using densitometry.

In contrast, cell cultures grown with GDNF showed an increase in the levels of both enzymes, especially that of superoxide dismutase (table 4.1). These results were confirmed by subsequent densitometric analysis of the membranes. An increase in the protein levels could stem from either an increase in the levels of catalase and SOD in the cells, or possibly a change in the proportion of neurons present in the cultures. To address this issue cell cultures grown for 36 hours with NT-3 and GDNF were stained using antibodies against catalase and SOD Cu/Zn and subsequently visualised using the Zeiss Axiophot microscope. As shown in Fig. 4.6, the relative intensity of catalase and SOD Cu/Zn staining does not appear to be different to the one observed in the cultures stained after 12 hours incubation with factors, which would argue against the upregulation of the levels of either catalase or superoxide dismutase.

On the other hand GDNF increased the relative number of the neurons present in the cultures as measured by cell counts of PGP 9.5 stained cultures fixed after 12 and 36 hours, especially in comparison to control cultures (Fig. 4.6, Table 4.2). Such an effect of GDNF could be due to induction of proliferation of precursor cells present in the cultures, or promotion of the survival of existing neurons, or both. This observation might suggest that the increase in the levels of catalase and superoxide dismutase detected by western blotting is caused by increased neuronal numbers present in the GDNF treated cultures compared to controls. However, data presented in Table 4.2 shows that the ratio of neurons to glial cells, and so their relative contribution to the total levels of both antioxidant enzymes detected by western blotting remains similar in the GDNF treated cultures both at 12 and 36 hours. This fact, in conjunction with western blots seems to indicate that GDNF is indeed able to upregulate the levels of both catalase and superoxide dismutase in the cultures of enteric ganglion cells.

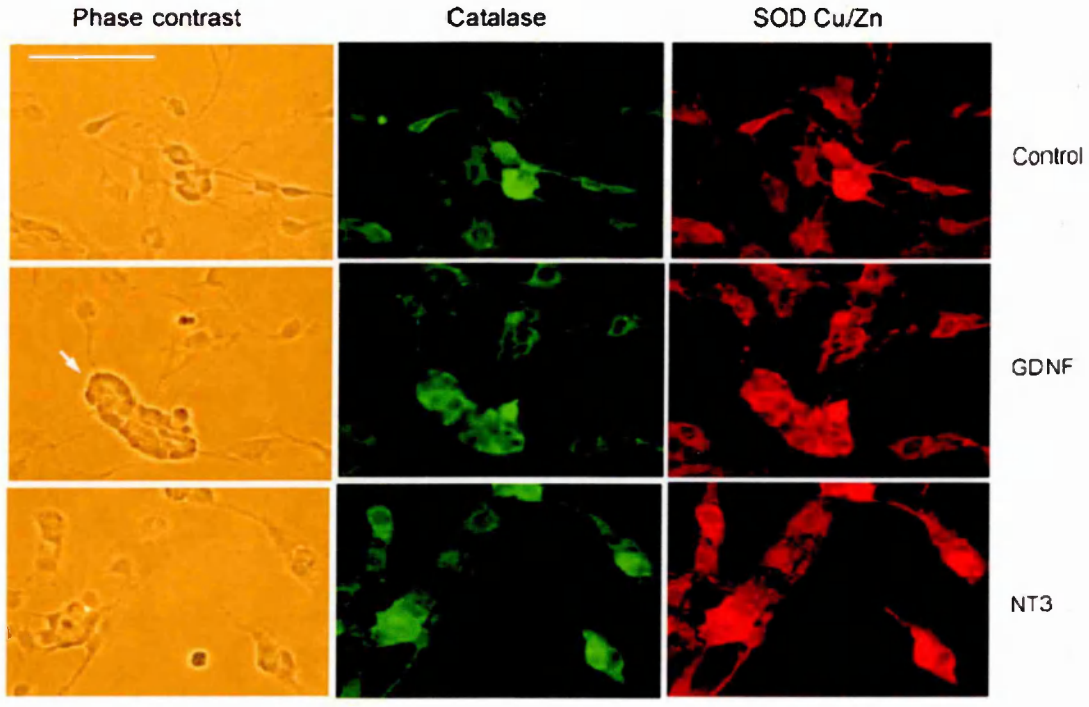


Figure 4.6 Immunohistochemical staining of cultures of enteric ganglion cells grown for 36 hours with NT-3 or GDNF. Each row shows the same field of view, pictures taken in phase contrast in the left row (yellow), middle row shows catalase immunoreactivity visualised by fluorescein (green), right row shows SOD Cu/Zn immunoreactivity visualised by rhodamine red (red). The staining intensity of catalase and SOD is comparable with cultures stained after 12 hours incubation with NT-3 and GDNF. Notice the group of neurons present in the GDNF treated culture (marked with a white arrow). Pictures taken at X400 magnification using the same exposure time. White bar at the top left panel represents 50 μm.

	Control		NT-3		GDNF	
	neurons	neurons/glia ratio	neurons	neurons/glia ratio	neurons	neurons/glia ratio
12 hours	159 (±0.36)	0.31	169 (±0.41)	0.36	176 (±0.55)	0.37
36 hours	119 (±0.32)	0.23	151 (±0.37)	0.29	195 (±0.44)	0.34

Table 4.2 Effect of factor treatment on the mean number of neurons, and the neuron to glial cells ratio in cultures of enteric ganglion cells after 12 and 36 hour incubation. Cultures established as described in materials and methods were grown in the presence of NT-3 or GDNF, both at 10ng/ml concentration, fixed after 12 or 36 hours and subsequently stained with PGP 9.5. Values represent the mean of neuronal numbers counted in 3 separate experiments, each treatment consisting of 3 replicas per experiment.
Values in parentheses represent ± SEM.

4.5 Discussion

There are data supporting the view that neurotrophic factors can affect the levels and activities of the enzymatic antioxidant defences both *in vivo* and *in vitro* [Chao et al. 1999, Guegan et al. 1999, Gabaizadeh et al. 1997]. This study concentrated on two of the antioxidant enzymes, SOD Cu/Zn and catalase, and tested changes in the protein levels of these enzymes in response to incubation of enteric ganglion cells with NT-3 and GDNF. Both SOD Cu/Zn and catalase participate in the metabolism of free radicals in cells. Cytosolic SOD Cu/Zn is a main enzyme utilized by cells to remove superoxide anions ($O_2^{\bullet-}$) arising as a by-product of electron transport chain. It is known that damage to mitochondria could result in malfunction of electron transport chain which in turn could cause an increased leakage of $O_2^{\bullet-}$ from mitochondria into the cell, therefore SOD Cu/Zn, as an enzyme participating in the removal of free radicals can contribute to increased survival of cells exposed to adverse conditions that impair mitochondrial function, such as oxidative stress.

In the model of oxidative stress used in this study, H_2O_2 was employed to induce oxidative stress. Since SODs are not able to use H_2O_2 as their substrate, the role of these enzymes in rescuing enteric ganglion cells subjected to H_2O_2 *in vitro* is probably limited to the removal of free radicals, such as superoxide anions arising in the cells as an effect of H_2O_2 interacting with cellular components. In the *in vivo* situation however, SODs are the first line of defence against superoxide radicals arising in mitochondria. Hence regulation of SOD activity and expression is important from a practical point of view, offering potential targets for therapies dealing with oxidative damage to various tissues.

On the other hand, catalase acts directly on H_2O_2. In an *in vivo* situation, H_2O_2 arising, among others, as a product of reaction catalysed by SODs is neutralized by catalase. In the experimental model used in this study, where H_2O_2 has been added directly into the culture medium, catalase would be one of the main enzymes responsible for dealing with deleterious effects of the oxidative stress.

The current set of experiments was designed to test the hypothesis that the neuroprotection offered by NT-3 observed in the previous chapter was due to an increase in the levels of antioxidant enzymes present in the cultured enteric ganglion cells.
The results of this study, however, showed that cultures of enteric ganglion cells grown for 12 or 36 hours in the presence of NT-3 do not exhibit significant increases of the levels of either catalase or SOD Cu/Zn. This suggests that the neuroprotective effect of NT-3 on enteric ganglion cells exposed to oxidative stress is not due to the effect of NT-3 on the levels of these two enzymes.

It is known that NT-3 acts as a survival factor for neurons at certain stages of their development [Davies et al. 1994]. Considering that fact, as well as the known role of NT-3 in the maintenance and survival of some of neuronal populations during adulthood it stands to reason that NT-3 might have protective effects on ENS ganglion cells facing adverse conditions, assuming they express appropriate receptors, which is the case for enteric ganglion cells used in this study (Dolatshed, Saffrey, personal communication). That, however, raises the question about the mechanism employed by NT-3 in protection of enteric ganglion cells exposed to oxidative stress. The hypothesis that NT-3 protects enteric ganglion cells exposed to oxidative stress by boosting their antioxidant defences is one of the possible explanations. If that is the case, then SOD Cu/Zn and catalase are two possible targets of NT-3: mitochondrial

SOD Mn, GPx, GR or glutathione are remaining ones. Although the results obtained here argue against SOD Cu/Zn and catalase as mediators of NT-3 neuroprotection against oxidative stress, it has been shown that another member of neurotrophin family, NGF, can increase activity of glutathione peroxidase [Guegan et al. 1999], which suggests that protective effect of NT-3 might also rely, at least partially, on that enzyme. Other studies suggest that increased levels of SOD Mn conveys increased protection against free radicals. To address this possibility, attempts were made to examine the changes NT-3 and GDNF treatment might have on the levels of this enzyme. However, no conclusions could be drawn due to the low specificity of the several anti SOD Mn antibodies tested, which leaves open the possibility of induction of SOD Mn expression in response to NT-3 treatment.

Except for its effects on the *level* of expression of antioxidant enzymes tested in this study, it is also possible that protective actions of NT-3 treatment could have been due to increased activity of catalase or SOD Cu/Zn. If that is the case, then such action of NT-3 might explain the increased neuronal survival in the cultures exposed to H_2O_2 and grown with NT-3, as described in chapter 3. However, more data are required to confirm or reject this hypothesis.

The second neurotrophic factor tested, GDNF, had a marked impact on protein levels of catalase and SOD Cu/Zn, although the effect was most pronounced at the later, 36 hour time point. This result might seem contradictory to the lack of protective effect observed in the experiments described in the previous chapter. Immunohistochemical staining of cultures grown for 12 and 36 hours with GDNF, as well as data on cell numbers offer an explanation for this observation. Since the effects of GDNF treatment on the levels of catalase and SOD Cu/Zn, as well as on cell numbers became most prominent after 36 hours exposure, it is possible that the factor treatment had not exerted its full effects in the experiments discussed

in chapter 3, where cultures were grown for just 12 hours with GDNF. Based on these results it seem safe to assume that the protective effects of GDNF treatment would be much more pronounced in the cultures exposed to H_2O_2 after GDNF treatment lasting 36 hours.

GDNF has been shown to be critical for the survival and proliferation of enteric precursor cells [Schafer et al. 1999], a similar effect of GDNF was also observed in this study, where GDNF increased cell numbers in the cultures. Cell counts of both Hoechst/propidium iodide and PGP 9.5 stained cells show an increase in both the glial and neuronal cell numbers after GDNF treatment (Fig. 3.5, Table 3.2 ,Table 4.2). This effect seems to continue throughout the time of the experiment as indicated by gradual increase of both neuronal and glial cell numbers with increased incubation times. This observation suggests that there are precursor cells present in the cultures, able to respond to GDNF treatment; a hypothesis supported by experimental data (A. Silva, personal communication).

Although cell cultures treated with GDNF showed increased neuronal and glial cell numbers compared to control or NT-3 treated cells, the proportion of neurons to glial cells remained constant between cultures grown for 12 and 36 hours. This observation, in conjunction with increased levels of catalase and SOD Cu/Zn observed in the cultures grown with GDNF for 36 hours implies that GDNF is able to increase the expression of both enzymes in the enteric ganglion cells *in vitro*, however more data are required to confirm this hypothesis.

Comparing the results of this chapter with the ones obtained in chapter 3, one has to bear in mind small differences in experimental design used in the two sets of experiments. While in chapter 3 the cultures were grown for 12 hours with appropriate factor prior to H_2O_2 exposure, experiments in this chapter have been focused primarily on the effects of NT-3 and GDNF of

the levels of antioxidant enzymes in the cultured enteric ganglion cells in the absence of H_2O_2. Although in both sets of experiments cultures were grown with factors for 12 hours, the H_2O_2 exposure might have caused changes in the levels or activities of antioxidant enzymes, different from the changes introduced by factor treatment alone. Although it is known that prolonged H_2O_2 exposure can induce expression of antioxidant enzymes in some cell types [Lai et al. 1996], its effects on the cells in the experiments described in this chapter and chapter 3 should be minimal, due to the short exposure time and rapid rate of decomposition of H_2O_2 in culture.

In summary, results of western blots and immunohistochemical staining of the cultures suggests that the neuroprotective effect of NT-3 on the enteric ganglion cells exposed to oxidative stress are not due to NT-3 induced changes of SOD Cu/Zn or catalase protein levels present in the cultures. These results also imply that the increased protein levels of these enzymes present in the cells treated with GDNF for 36 hours may be due to the increased expression of catalase and SOD Cu/Zn in the cells present in these cultures, as demonstrated by cell count data and western blot analysis. Alternative mechanisms of NT-3 induced neuroprotection are investigated in chapter 5.

Chapter 5

Signalling pathways activated by NT-3 exposure in enteric ganglion cells *in vitro*

Summary

The experiments described in this chapter were undertaken in order to examine the signalling events initiated by NT-3 and GDNF treatment in enteric ganglion cells *in vitro*. Cell cultures grown with NT-3 or GDNF were treated with the phosphoinositide-3 kinase (PI-3K) inhibitor, LY294002. Inhibition of PI-3K resulted in drastic decrease of both neuronal and glial cell numbers after 12 hours, regardless of factor treatment. This effect was even more pronounced after 36 hours. Moreover, inhibition of PI-3K nullified the effects of NT-3 and GDNF treatment. These results imply that PI-3K is involved in the survival of cultured enteric ganglion cells, as well as participating in signalling events initiated by both NT-3 and GDNF.

Western blots performed on the cultures treated with NT-3 showed constant high levels of phosphorylated Akt protein present in these cells, independent of factor treatment.
In contrast, levels of phosphorylated MAPK/ERK kinase (MEK) and extracellular regulated kinase (ERK) proteins increased in response to NT-3 stimulation. Total protein levels of bcl-2 and c-fos proteins were not affected by NT-3 treatment.

Combined NT-3 and LY294002 treatment significantly decreased the amount of phosphorylated Akt protein in the cultures. Upon addition of NT-3, a minor increase in the amount of phosphorylated Akt occurred, although not reaching the levels observed in the uninhibited cultures. The increase lasted until 20 minutes after NT-3 stimulation, with subsequent decline. LY294002 treatment did not affect the phosphorylation of either MEK or ERK, however levels of phosphorylated forms of both proteins peaked 20 minutes after NT-3 addition and declined afterwards, similar to Akt. In contrast to Akt, MEK and ERK, inhibition of PI-3K caused an increase of total protein levels of bcl-2 and c-fos proteins.

5.1 Introduction

As discussed in the general introduction (chapter 1, section 1.4.2) neurotrophins regulate growth, development, survival and repair responses of the nervous system [Yamamori et al. 1992, Cowen et al. 1998]. All these actions are initiated by binding of neurotrophins to their receptors, the Trk tyrosine kinase receptors and the p75 neurotrophin receptor [Bothwell et al. 1995]. These receptors can either collaborate with each other, or inhibit each other's actions; such functional interplay of Trk and p75 generates signals affecting neuronal behaviour. Interestingly, while Trk receptors transmit positive signals, such as enhanced neuronal survival and growth, p75 can transduce both positive and negative signals [Chao et al. 1994].

Our understanding of the signalling pathways involved in regulation of neuronal behaviour has grown considerably over last couple of years, with every year bringing discovery of new signalling molecules. Although complex, some of the mechanisms responsible for neuronal survival as well as differentiation responses have been elucidated in a degree allowing us to identify and describe key molecules in a given pathway, as well as their interactions. Recent investigations have started to unveil the fact that Trk/p75 signalling is regulated by connecting a variety of intracellular signalling cascades which include proteins encoded by proto-oncogenes and tumor suppressor genes. To date, several intracellular signalling proteins and signal transduction pathways used by Trk and p75 have been identified. The first molecule shown to be activated by neurotrophins was Ras, a small GTP-binding protein. Although Ras is responsible for most of the survival responses initiated by neurotrophins, it does not act directly to promote survival, instead translating and directing signals initiated by neurotrophins into different signalling pathways.

Two major pathways that play a role in the neurotrophin mediated signalling are the PI-3K /Akt and MEK/MAPK pathways, involved among others in survival, neurite outgrowth and differentiation responses. Both signalling molecules, as well as the pathways, are described below.

5.1.1 Signalling cascades initiated by the activation of Trk receptors

As mentioned above, Trk receptors initiate signalling cascades which follow two major pathways, the Ras/PI-3K/Akt and the MEK/MAPK/ERK pathways, shown in figure 5.1. Both cascades are initiated by binding of neurotrophins to their respective receptors, which in turn stimulates receptor transphosphorylation that results in recruitment of a series of signalling proteins to docking sites on the receptor. These proteins include Shc, which activates Ras through adaptor proteins Grb-2 and SOS [Kaplan et al. 1997], among others. At the point of Ras the signalling pathway forks, leading to the activation of the MEK/MAPK or PI-3K/Akt pathways.

The PI-3K pathway is activated by Ras, in turn activated by a group of adapter molecules [Yamada et al. 1997]. PI-3K stimulates the activities of many signalling proteins, among those proteins modulating execution of cell death program. It has been shown that some anti-apoptotic proteins have been induced in chick sensory and sympathetic neurons in a manner dependent on PI-3K activity [Wiese et al. 1999].

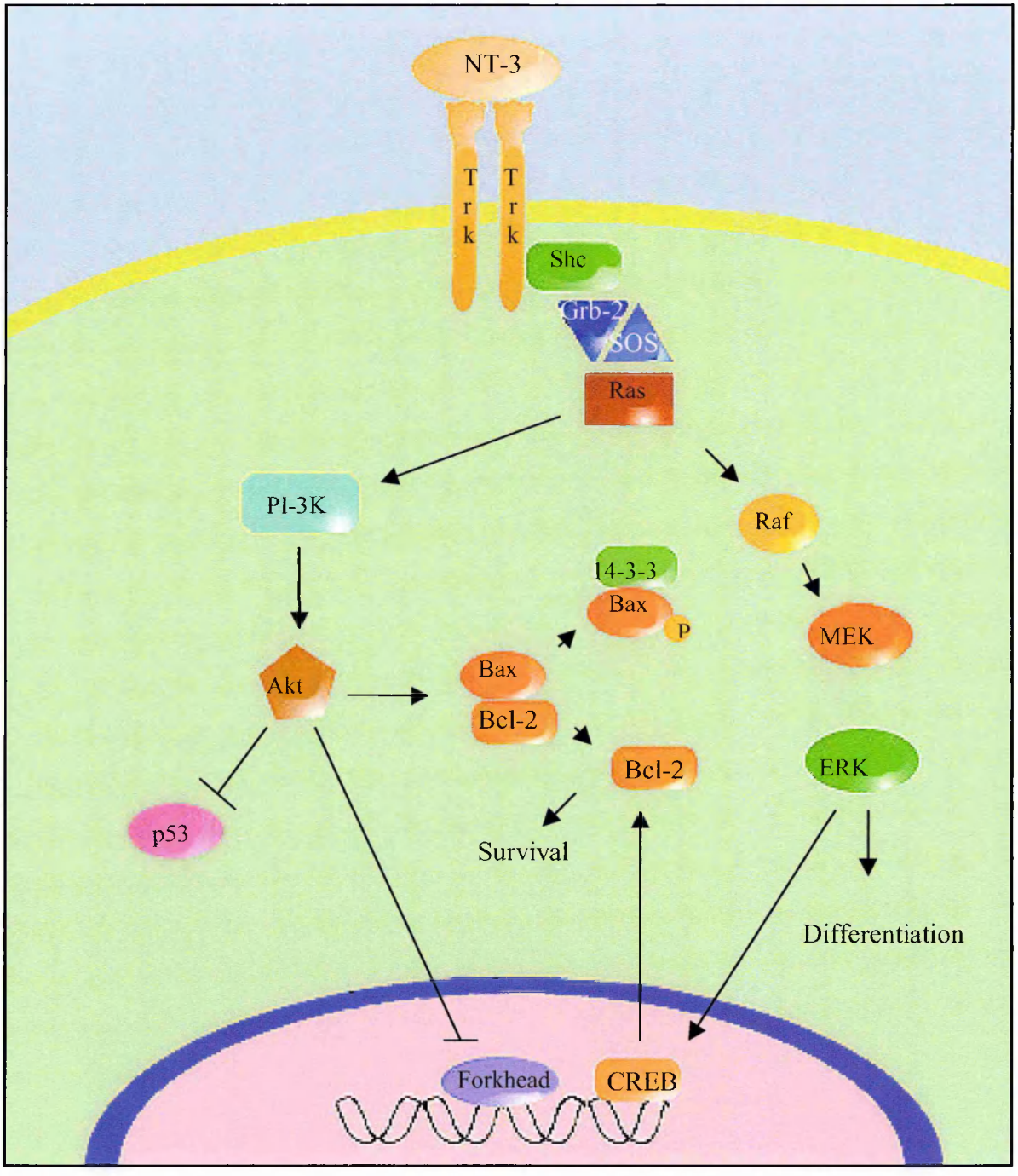


Figure 5.1 An outline of two major pathways responsible for inducing survival responses in neuronal cells, activated by Trk receptors. Binding of ligand to Trk stimulates its phosphorylation, with subsequent recruitment of several auxiliary proteins. Signalling pathways diverge at Ras protein, which, depending on additional signalling events could direct it into PI-3K pathway or MEK/ERK pathway. Both pathways are discussed in more detail in the text.

One of major downstream effectors of PI-3K is the serine/threonine kinase Akt, activated by PI-3K in response to NGF stimulation [Andjelkovic et al. 1998]. Akt acts on several target molecules; interestingly many proteins acted on by Akt have pro-apoptotic properties, and are inactivated as a result of Akt stimulation [Datta et al.1999], which is the main mechanism by which PI-3Kthe /Akt pathway conveys its survival effects. One of the first reported targets of pro-survival activity of Akt was pro-apoptotic protein Bad

Phosphorylation by Akt has been found to induce association of Bad with anti-apoptotic protein 14-3-3, and prevented Bad from forming a complex with anti-apoptotic Bcl proteins, which would inactivate them [Datta et al. 1997]. Evidence for the importance of Akt induced inactivation of Bad comes from overexpression experiments in cerebellar neurons, where insulin-like growth factor 1 (IGF-1) or constitutively active Akt suppressed the apoptotic activity of wild type Bad, but not of Bad mutated at the site phosphorylated by Akt [Datta et al. 1997].

Another protein target of Akt in neurons is a transcription factor Forkhead 1 (FKHRL 1). Genetic studies in *C. elegans* indicated that the activity of a forkhead family member, DAF 16, which contains an Akt consensus phosphorylation site, was suppressed by Akt [Paradis et al. 1998]. It has also been shown that expression of FKHRL1 mutated at Akt phosphorylation sites increased apoptosis of cerebellar neurons cultured in IGF-1 [Brunet et al. 1999].

Data available to date on actions of Akt indicate that it may suppress apoptosis directly by inhibiting the activities of pro-apoptotic proteins Forkhead or Bad, increasing levels of anti-apoptotic proteins such as Bcl-2 or by blocking the apoptotic pathway in neurons.

All these results suggest that a signalling pathway consisting of Ras/PI-3K/Akt is the major regulator of neuronal survival.

Although Akt is an important downstream effector of PI-3K activity, it is most probably not the only target of PI-K induced survival signalling, since inhibition of PI-3K is often more effective than inhibition of Akt at suppressing survival responses [Grewal et al. 1999]. A second pathway used by the neurotrophins consists of the Ras/MEK/MAPK pathway. The cellular responses initiated by this pathway include synaptic plasticity, long-term potentiation, survival and others. The major survival effect of this pathway is most likely protection of neurons from death due to injury or toxicity, rather than from trophic factor withdrawal.

For example, in cortical neurons constitutively active MEK protected, and inhibition of MEK blocked BDNF regulated neuroprotection from campothecin induced apoptosis [Hetman et al. 1999]. Similarly, MEK/MAPK protected sympathetic neurons against apoptosis due to cytosine arabinoside [Anderson et al. 1999] and cerebellar neurons from apoptosis caused by oxidative stress [Skapner et al. 1998].

The effects of the MEK/MAPK pathway are achieved by stimulating the activity or expression of anti-apoptotic proteins, including the transcription factor CREB (cAMP response element binding protein) and Bcl-2. CREB is a mediator of neuronal survival, as dominant-inhibitory forms of CREB induced apoptosis of virtually all sympathetic neurons grown with NGF [Riccio et al. 1999], and also some neurons grown in BDNF. The activation of CREB by survival factors is likely to be due to phosphorylation by multiple kinases, including the MAPK activated kinase Rsk [Bonni et al. 1999].

NGF strongly increased Bcl-2 levels in sympathetic neurons, which in turn protected these and other neurons from apoptotic cell death [Michaelidis et al. 1996]. Inhibition of MEK/MAPK activity in PC12 cells completely blocked the ability of NGF to increase Bcl-2 levels [Liu et al. 1999], suggesting that Bcl-2 is a transcriptional target of the MEK/MAPK pathway. Since CREB activity is also required for Bcl-2 expression and survival induced by NGF in sympathetic neurons, it suggests that all these proteins might form a survival pathway for sympathetic neurons leading from Ras, through MEK and MAPK to CREB and Bcl-2.

In spite of the differences between neurotrophins and the GDNF family of neurotrophic factors, both employ similar intracellular signalling mechanisms. It has been shown that GDNF-induced Ret signalling activates both PI-3K/Akt and MEK/MAPK pathways [Chen et al. 2001], employing adaptor and effector proteins that also participate in neurotrophin-induced signalling events [Takahashi et al. 2001].

5.1.2 Overview of signalling molecules involved in Trk/p75 receptor signalling

5.1.2.1 Ras

The Ras family was first identified as products of viral genes responsible for tumor formation in mice and rats [Kristen et al. 1967]. Ras proteins function as molecular switches determined by whether they are bound to guanine diphosphate (GDP), which is 'off' position or guanine triphosphate (GTP), the 'on' position. Inactive, GDP-bound Ras proteins are activated by interaction with members of large and structurally diverse class of proteins termed guanine-

nucleotide exchange factors (GEF's), which catalyse the release of GDP, which is replaced by GTP [Lenzen et al. 1998].

One of the best characterized signal transduction pathways downstream of p21 Ras is the one that leads to activation of members of the mitogen-activated protein (MAP) kinase family, especially Erk 1/2, which requires binding of Ras to members of the Raf family of serine/threonine kinases [Vojtek et al. 1993, Zhang et al. 1993].

Another effector of p21 Ras is phosphatidylinositol-3 kinase (PI-3K). Its catalytic subunit interacts directly with GTP-bound Ras [Rodriguez et al. 1994]. Some members of Ras family might increase activity of PI-3K more efficiently than others [Marte et al. 1997, Yan et al. 1998].

5.1.2.2 Phosphatidylinositol-3 kinase (PI-3K)

The PI-3 kinases are a family of enzymes that phosphorylate the hydroxyl group of phosphoinositides. Based on sequence homology and substrate preference, PI-3Ks have been divided into 3 classes, of which class I is mostly involved in transducing signals initiated by binding neurotrophins to Trk receptors.

Class I PI-3Ks regulate many cellular functions, including apoptosis, cellular proliferation, vesicular trafficking, cytoskeletal structure and cellular morphology, glucose utilization, protein biosynthesis and lipid metabolism. Enzymes of this class

consist of heterodimeric enzymes composed of a catalytic subunit and an adaptor/regulatory subunit [Chan et al. 1999]. There are two major subclasses of the catalytic subunit, Ia and Ib. Both subclasses contain carboxy-terminal catalytic and phosphatidylinositol kinase domains and an amino-terminal Ras binding domain. There are also two main regulatory subunits, containing two carboxy-terminal Src homology-2 (SH2) domains, as well as amino-terminal proline binding SH3 domain.

The biological significance of the multiple catalytic and regulatory subunits is not completely understood. All the regulatory subunits can associate with and recruit all the catalytic subunits to proteins containing appropriate motifs which contain phosphorylated tyrosine. The functional overlap between these molecules is further supported by genetic studies in mice. Mice homozygous for the targeted disruption of the entire regulatory subunit gene die perinatally [Fruman et al. 1999], while knockout mice that express amino-truncated versions of this protein survive the perinatal period [Suzuki et al. 1999].

Some of the differences regarding the biological roles of these proteins could be caused by differences in their patterns of expression in different organs and cells. For example, while some catalytic subunit isoforms are widely expressed, expression of others is restricted to certain cell types [Vanhaesebroeck et al. 1997].

5.1.2.3 Akt

Akt, also known as protein kinase B (PKB) was originally identified as a viral oncogene. At present, three members of the Akt family have been identified. Although they are products of different genes, they are highly related, showing more than 80% sequence homology [Nicholson et al. 2002]. The Akt family of kinases is evolutionarily conserved in all eukaryotes.

Inactive Akt localizes in cytosol; it is recruited into the plasma membrane by the lipid products of PI-3K in response to a variety of stimuli, including hormones, growth factors and cytokines. Subsequently Akt undergoes phosphorylation which results in its activation [Nicholson et al. 2002].

In neurons, Akt has only been shown to regulate survival, and not any other responses such as neurite outgrowth or differentiation. Thus all of the proposed direct targets of Akt identified so far have been proteins that regulate cell survival, such as Bad (inhibitor of anti apoptotic protein Bcl-2), pro-caspase 9 and forkhead, a transcription factor inducing apoptosis, among other things. In each case Akt suppresses apoptosis by phosphorylating the apoptotic protein at the Akt consensus phosphorylation site.

Data gathered to date indicate that Akt plays a role in the survival of cerebellar neurons induced by IGF-1 treatment [Dudek et al. 1997] as well as survival of sympathetic neurons induced by NGF [Virdee et al. 1999]. Akt not only mediates growth factor regulated cell survival, but also neuronal survival promoted by depolarisation [Crowder et al. 1999].

5.1.2.4 MAP kinases

The mitogen activated protein kinases (MAP kinases) comprise a family of related serine-threonine protein kinases that regulate signalling leading to a variety of cellular responses ranging from apoptosis and oncogenic transformation to inflammatory responses. Components of the MAP kinase pathways are evolutionarily highly conserved in structure and organisation, each consisting of a module of three cytoplasmic kinases: a mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK, eg. Raf), MAP kinase kinase (MAPKK, eg. MEK) and the MAP kinase, eg. ERK (Fig. 5.2). The first member of the pathway is MAPKKK, which is a serine-threonine kinase that receives activating signals from a transmembrane receptor and then phosphorylates and activates its substrate, MAPKK. MAP kinases have the potential to phosphorylate other cytoplasmic proteins, as well as translocate from cytoplasm to the nucleus, where they directly regulate the activity of transcription factors controlling gene expression.

The best understood MAPK signal transduction pathway of mammalian cells is the one formed by the Raf/MEK/ERK kinases. Proliferative signals induced by growth factors cause activation and autophosphorylation of their cognate receptor tyrosine kinases. The phosphorylated tyrosine residues of the receptor serve as docking sites for the adaptor proteins, which activate Ras, which in turn recruits the MAPKKK protein Raf to the plasma membrane where it is activated. The precise mechanism of Raf activation is not clear, but activated Raf signals via MEK to ERK [Daum et al. 1994].

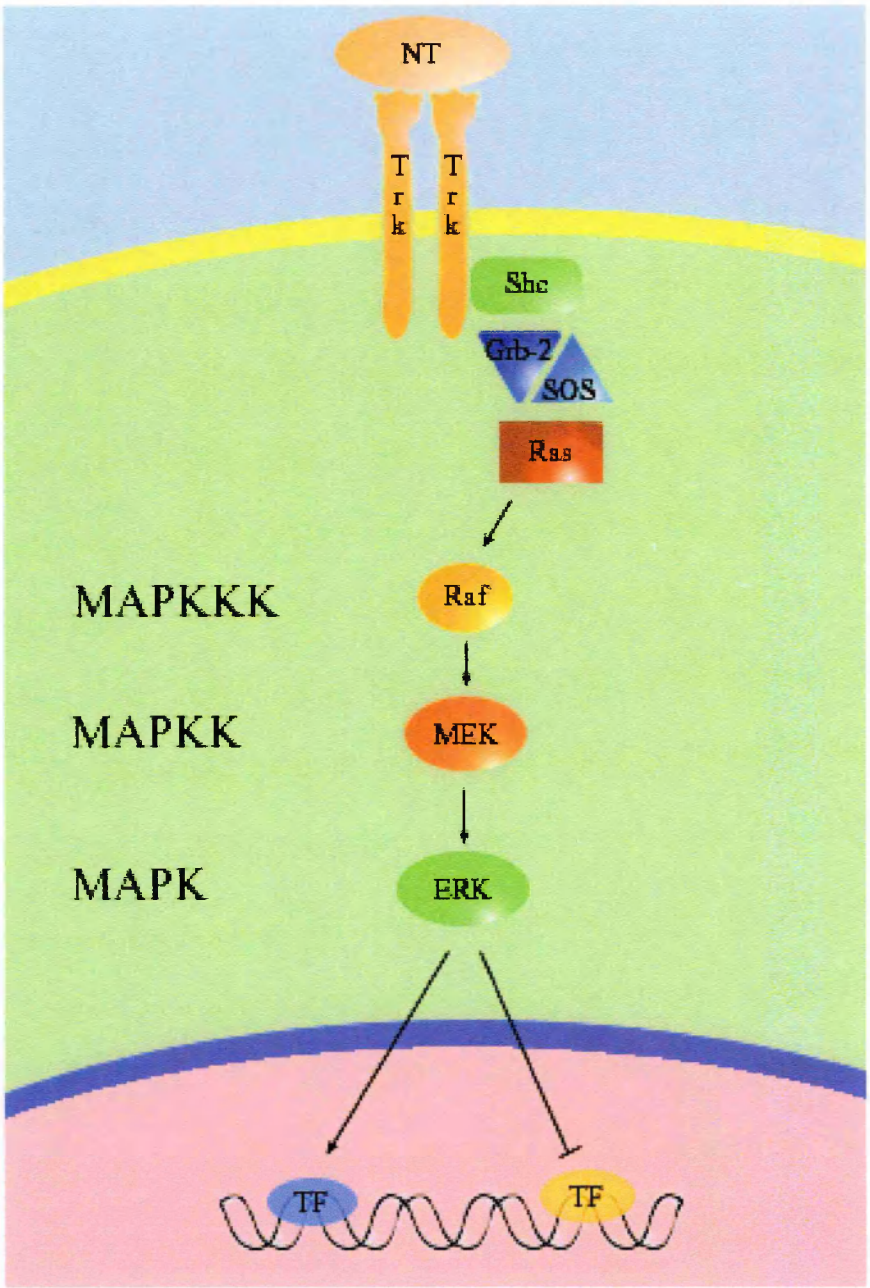


Figure 5.2 Schematic representation of MEK/ERK signalling pathway in mammalian cells. The activity of this pathway affects a range of different transcription factors (TF), either by repressing, or by stimulating their activity. For more details see text.

Activation of MEK by Raf involves phosphorylation on two serine residues, which subsequently leads to phosphorylation of ERK by MEK. Such a mode of signalling amplifies the signal; there are some data suggesting that activation of only small number of Ras molecules is sufficient to induce full activation of ERK [Hallberg et al. 1994]. Activated ERK phosphorylates its substrates localized in practically all cellular compartments [Lewis et al. 1998].

There are two isoforms of ERK; ERK1 and ERK2. Their specific roles are not yet fully understood. Both isoforms are widely expressed, and share large degree of homology in their amino acid sequence as well as similar substrate specificity. However, knockout of one of the isoforms produces different results. ERK1 null mice are viable, fertile and of normal size [Pages et al. 1997].

In these animals ERK2 can compensate for most of the functions of ERK1, with the exception of thymocyte maturation which is impaired [Traverse et al. 1992]. On the other hand, ERK2 knockout is lethal at early embryonic stages, about day 6.5 [Pouyssegur et al. 2002]. Considering the pleiotropic substrates and the ubiquitous expression of ERK, cell specific regulation must occur to ensure transduction of cell-specific signals. For example, expression of one of the ERK regulator proteins is restricted to only a few cell types, such as terminally differentiated astrocytes [Kahan et al. 1992].

In a single cell, activation of the ERK pathway can lead to antagonistic effects, for example in PC12 cells both differentiation after NGF treatment and cell proliferation resulting from EGF stimulation require ERK activation. In PC12 cells EGF causes transient activation of ERK, while NGF promotes sustained activation of ERK, hence the duration of ERK activation

specifies cellular response [Traverse et al. 1992]. It has also been shown that strong ERK activation protects cells from apoptosis induced by anchorage and serum removal, whereas moderate activation of ERK is necessary to permit apoptosis.

5.1.2.5 c-fos

c-Fos can be activated by many signalling cascades, among those the PI-3K/Akt and the MEK/ERK described above. The Fos (AP-1) family consists of the transcription factors, which form homodimers or heterodimers with each other and which bind to a specific DNA sequence called AP-1. Fos proteins are a nuclear proteins, with DNA-binding and gene activator properties [Sambucetti et al 1986]. They are also functionally related to c-Jun proteins [Rausher et al. 1998].

Because of the structural differences, c-fos cannot form homodimers the way c-jun does [Neuberg et al. 1989], and c-fos proteins cannot bind to DNA unless they dimerize with jun proteins [Chiu et al. 1988]. However, c-fos enhance DNA binding in these dimers due to the fact that some of the jun proteins are unable, or able to only weakly bind DNA.

The convergence of pathways on the c-fos promoter has been extensively studied *in vitro* and *in vivo*. The second messenger pathways include those activated by neurotransmitters and hormones, and the Ras- and mitogen stimulated pathways which are particularly effective in initiating c-fos expression [Karin et al. 1995, Morgan et al. 1991]. In neuronal PC12 cells and other neuronal species *in vitro*, the expression of c-fos can be induced by many agents, for example neurotransmitters such as acetylocholine or growth factors such as NGF and insulin

[Bartel et al. 1989, Curran et al. 1985]. In the case of growth factors, induction of c-fos occurs after ligand binding to membrane-bound tyrosine kinase receptors [Karin et al. 1994]. Inhibition of phosphorylation PI-3K in rat 3T3 cells blocks the insulin-induced synthesis of DNA and c-Fos by about 85% [Jhun et al. 1994].

Degradation of c-fos is governed by two instability domains within its transcript [Chen et al. 1994]. Depending on these sequences the half-life of c-fos is about 15 minutes, but saturation of degradation machinery when there are high concentrations of c-fos extends this time to 100 minutes. The c-fos/jun dimers binding to specific DNA sequences stimulate expression of a range of proteins. In neurons, these include, but are not restricted to neurotransmitter receptors, ion channels, cytoskeletal structures as well as proteins required for neurotransmitter synthesis [Herdegen et al. 1998]. Also there are data linking c-fos to developmental apoptosis [Smeyne et al. 1993].

5.1.2.6 bcl-2

Bcl-2 was initially shown to inhibit cell death induced by deprivation of interleukin 3, and subsequently shown to inhibit cell death induced by other stimuli including chemotherapeutic agents and heat shock [Tsujimoto et al. 2000]. In recent years, it has been established that Bcl-2 prevents most forms of apoptotic cell death as well as some forms of necrotic cell death. A large number of Bcl-2 related proteins have been isolated and divided into 3 major groups [Adams et al. 1998].

The first group is formed by anti-apoptotic members of the family, such as Bcl-2, which exert anti cell death activity. Proteins from this group also share a sequence homology, especially within four domains called Bcl-2 homology (BH) 1to 4, responsible for the dimerization, channel formation as well as other activities. The second group is formed by pro-apoptotic proteins such as Bax, which share sequence homology in BH1, 2 and 3, but not in BH4 domain. Finally the last group comprises of proteins which have pro-apoptotic function, such as Bik and others, which share homology only within BH3 domain.

One of the unique features of the Bcl-2 family proteins is heterodimerization between anti apoptotic and pro-apoptotic proteins, which is thought to inhibit the biological activity of the proteins forming the heterodimer. Formation of heterodimer is accomplished by insertion of BH3 region of a pro-apoptotic protein into a hydrophobic pocket present on the anti-apoptotic protein [Sattler et al. 1997]. In addition to the regulation of apoptosis by heterodimerization of anti-apoptotic and pro-apoptotic proteins from the Bcl-2 family, some members of the Bcl-2 family have been suggested to regulate apoptosis independently of each other. These observations are consistent with the findings that some Bcl-2 proteins, such as Bcl-2, Bcl-x_L and Bax can form channels in synthetic lipid membranes [Schendel et al. 1997, Schlesinger et al. 1997]. Among other functions, proteins of the Bcl-2 family also play a key role in controlling the activation of caspases: enzymes involved in the execution of cell death program [Korsmeyer et al. 1999], by controlling the release of cytochrome c which is one of the events required for initiation of apoptosis. Proapoptotic proteins, such as Bax and Bak increase mitochondrial permeability, allowing cytochrome c to pass into the cytoplasm, whereas antiapoptotic members: Bcl-2 and Bcl-x_L prevent release of cytochrome c. In addition, Bcl-2 is able to regulate activation of the caspase cascade independently of cytochrome c [Krebs et al. 1999]. Bcl-2, as well as the other proteins described above,

participates in signalling events initiated by the neurotrophic factors binding to their receptors. Although there is a considerable amount of data on the intracellular mechanisms employed by neurotrophic factors in numerous cell types, available data regarding these events in the enteric neurons is sparse. The aim of this study was to investigate the signalling events initiated by neurotrophic factors in the enteric neurons and glial cells.

5.2 Aims

The experiments described in this chapter were performed to investigate the signalling pathways involved in the molecular mechanisms underlying the effects of NT-3 observed in previous chapters. Preliminary experiments were designed to examine the role of PI-3K in the survival of the cultured enteric ganglion cells grown with NT-3 and GDNF. PI-3K activity was blocked using LY294002 inhibitor in the cultures and cell numbers were counted afterwards. The second set of experiments focused on the short term effects of NT-3 or NT-3 and LY294002 treatment on the level of the phosphorylated forms of several key signalling molecules including MEK, ERK and Akt, as well as total protein levels of c-fos and bcl-2. Western blots were performed on cell lysates obtained from NT-3 treated or factor and LY294002 treated cultures, using antibodies against phosphorylated forms of these proteins excluding c-fos and bcl-2, where total protein levels were examined.

5.3 Methods

The methods used in this chapter were described in chapter 2 (sections 2.4.1, 2.5, 2.6.1 and 2.7)

5.4 Results

5.4.1 The effects of PI-3K inhibition on the survival of enteric ganglion cells grown for 12 and 36 hours.

The two main pathways activated by neurotrophic factors in neurons are the PI-3K/Akt and the MEK/ MAPK pathways, responsible for modulating cellular responses to trophic factor stimulation. Experiments described in previous chapters focused on the general responses of enteric neurons to NT-3 and GDNF. The aim of this study was to examine molecular mechanisms underlying the effects of NT-3 and GDNF.

A preliminary set of experiments was designed determine whether PI-3K plays a role in the NT-3 and GDNF induced survival of enteric ganglion cells. To examine the role of PI-3K, cultures of enteric ganglion cells were established as described in materials and methods, and grown without factors or in the presence of NT-3 or GDNF with or without the specific inhibitor of PI-3K, LY294002 (300μM) for 12 and 36 hours. After these times the cultures were fixed, and neurons and glial cells were identified following staining with PGP 9.5. Cells were subsequently counted using a Zeiss microscope under 400X magnification.

The effect of PI-3K inhibition on cell numbers and cell morphology

In the cultures which did not receive factors, inhibition of PI-3K was observed to have a marked effect at the 12 hour time point, and its effects were much more prominent after 36 hours. Both neuronal and glial cell numbers were decreased compared to controls (see figures 5.4 and 5.5).

A similar effect was observed in NT-3 and GDNF treated cultures; a typical morphological response observed in the cultures treated with NT-3 and GDNF, that is neurite outgrowth caused by NT-3 and formation of neuronal cell clusters initiated by GDNF were reduced as a result of PI-3K inhibition (Fig. 5.3). After 36 hours incubation with LY294002, its effects were even more pronounced, with a drastic decrease of both neuronal and glial cell numbers, as well as the abolition of the morphological responses initiated by NT-3 and GDNF treatment (Fig. 5.4). In addition, some cells in the cultures treated with LY294002 showed signs of apoptotic cell death, that is condensation of chromatin and membrane blebbing at 12 hours time point; their number increased at 36 hours time point (not shown).

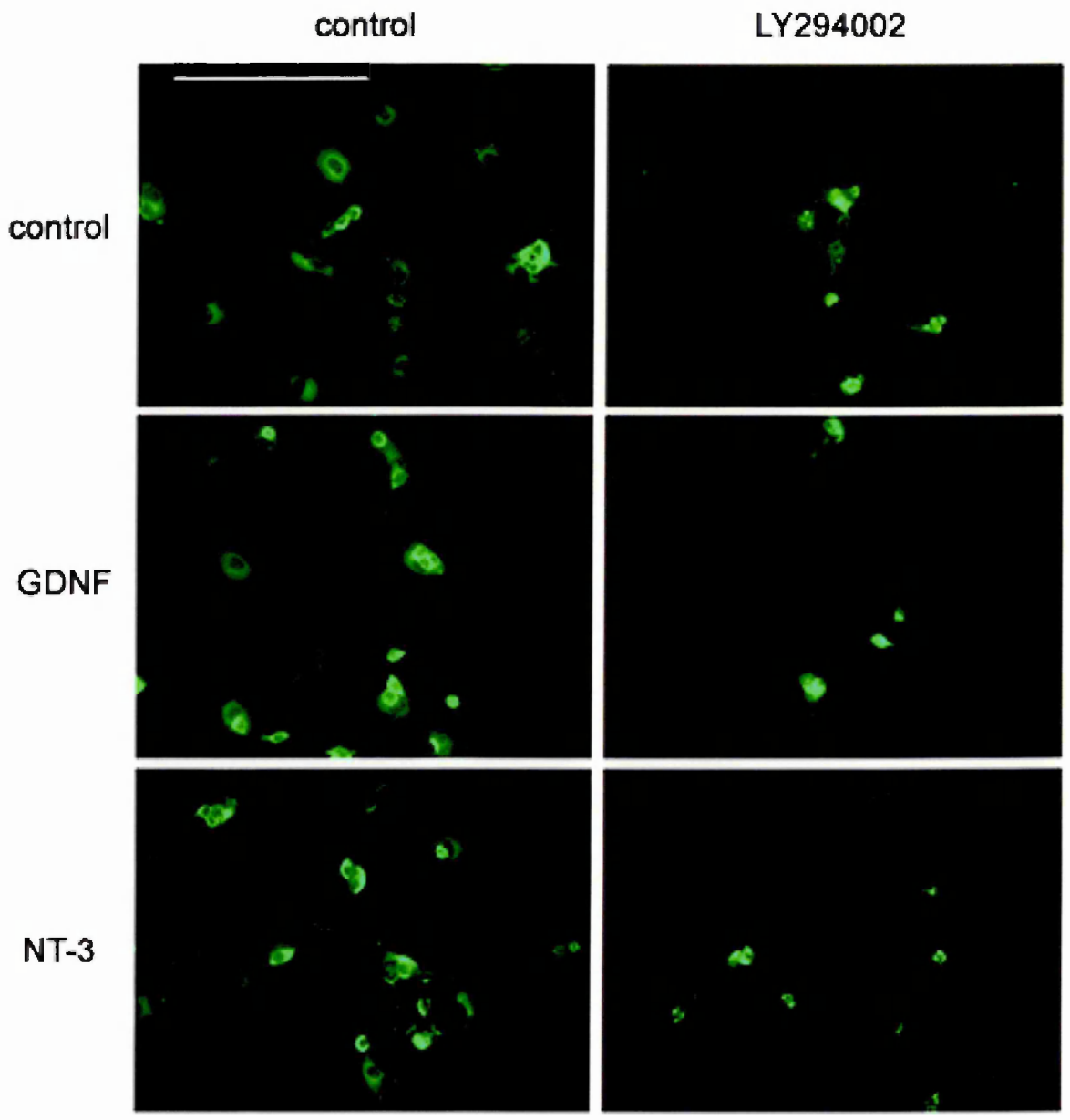


Figure 5.3 PGP 9.5 staining of cultured enteric ganglion cells grown for 12 hours without (left panel) or with LY294002 inhibitor (right panel). Control cells grown without inhibitor, as well cultures that received factor treatment display morphologies typically observed at this period. Note the group of neuronal cells present in the GDNF treated culture, as well as processes grown by neurons after NT-3 treatment. In contrast, LY294002 decreased cellular numbers in every treatment, as well as partially abolished the effects of both neurotrophic factors, as shown by lack of neuronal processes in the NT-3/LY294002 treatment and decreased number of neuronal cell clusters in GDNF/LY294002 treatment. Pictures taken under 400X magnification, white bar at the top left picture represents 50μm.

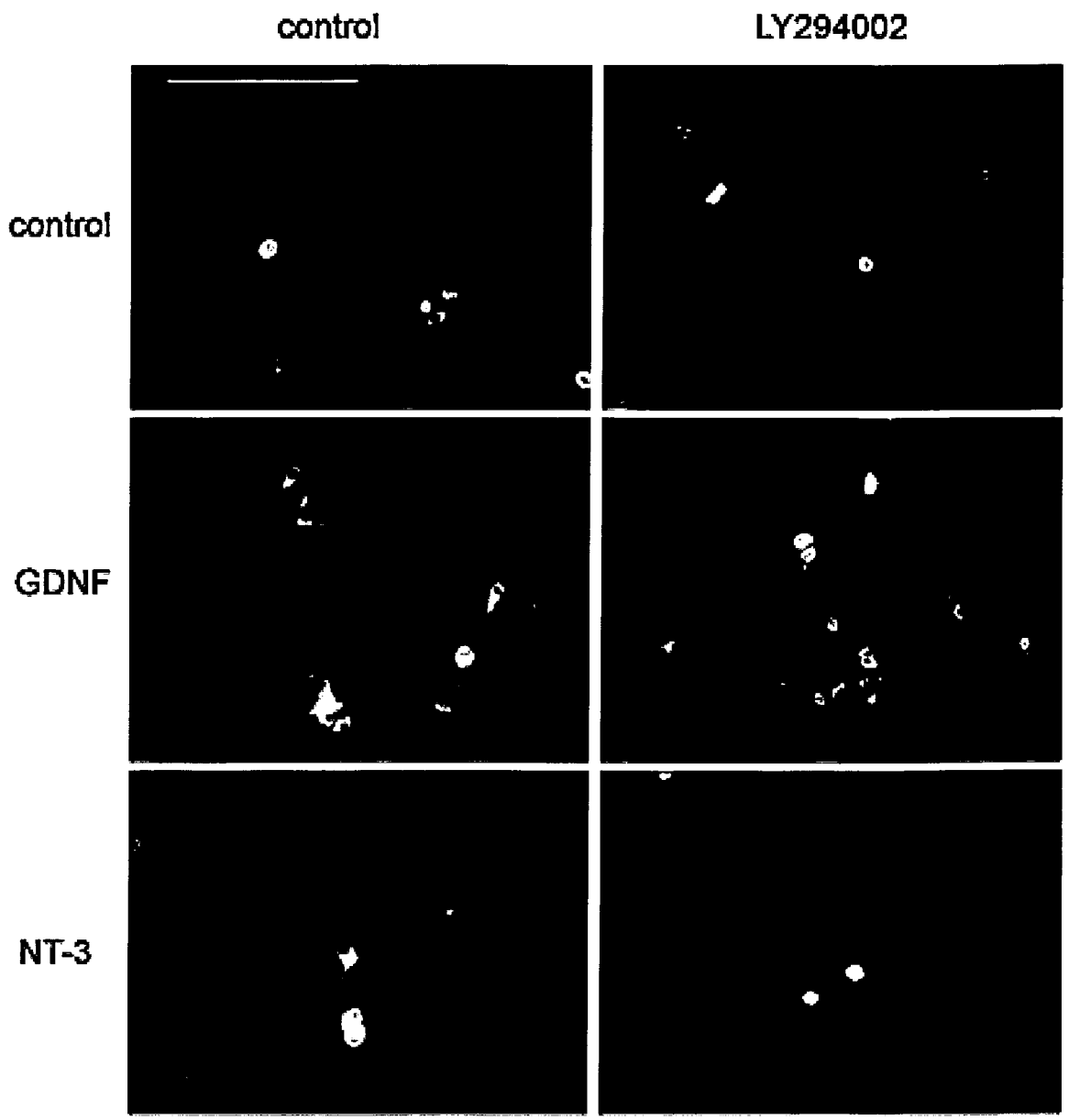


Figure 5.4 PGP 9.5 staining of cultured enteric ganglion cells grown for 36 hours without (left panel) or with LY294002 inhibitor (right panel). Again, cells grown without inhibitor display typical morphologies observed at this time period. Cultures incubated with GDNF for 36 hours contain a large number of neuronal cells forming clusters, while NT-3 treatment promotes formation of long processes by neuronal cells. The effects of PI-3K inhibition are much more pronounced at 36 hours time point. Both neuronal and glial cell numbers were significantly decreased, and morphological responses initiated by factor treatment also were very limited. Pictures taken at 400X magnification, white bar at the top left panel represents 50μm.

Analysis of cell numbers

Cell counts of the cultures confirmed the visual observations as summarized in Table 5.1 and Figures 5.5, 5.6, 5.7 and 5.8.

12 hours	Control		NT-3		GDNF	
	neurons	glia	neurons	glia	neurons	glia
Control	159 ±0.69	501 ±0.36	189 ±0.67	462 ±0.41	176 ±0.52	468 ±0.33
LY294002	69 ±0.73	256 ±0.33	58 ±0.72	244 ±0.32	60 ±0.72	242 ±0.33
36 hours						
Control	119 ±0.70	509 ±0.30	151 ±0.72	512 ±0.87	195 ±0.75	564 ±0.44
LY294002	30 ±0.55	87 ±0.31	25 ±0.37	89 ±0.34	40 ±0.70	101 ±0.43

Table 5.1 The effect of LY294002 inhibition on cultures of enteric ganglion cells grown without factors and with NT-3 or GDNF for 12 and 36 hours. Both factors were used at 10ng/ml. LY294002 was used at 300μM. Cultures grown for the designated times were fixed with 4% paraformaldehyde, stained against the pan neuronal marker PGP 9.5 and subsequently counted using a Zeiss microscope at X400 magnification, as described in chapter 2, section 2.5. Data presented are the average number of neurons and glial cells counted in 3 separate experiments, 4 replicas of each treatment per experiment. Values to the right of cell numbers represent ±SEM

Analysis of glial cell numbers present in the cultures after 12 hours incubation with LY294002 showed a significant, approximately 50% decrease compared to respective uninhibited cultures (table 5.1, Fig. 5.5). The effects of PI-3K inhibition on glial cell numbers differed slightly between cultures grown with and without factors. However, the differences were not statistically significant.

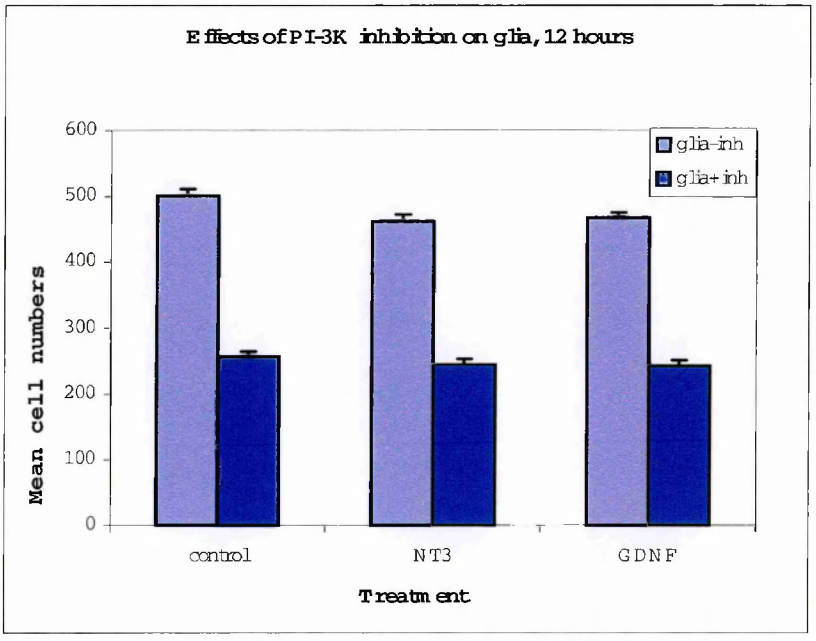


Figure 5.5 The effects of PI-3K inhibition on mean glial cell numbers after 12 hours incubation. Data are presented as a mean of 3 separate experiments, each treatment done in 4 replicas in each experiment. Error bars represent ± S.E.M.

Neuronal numbers in the cultures grown without factors decreased by 56% after 12 hours incubation with LY294002, compared to uninhibited counterparts. Both NT-3 and GDNF treated cells displayed an approximately 65% reduction of neuronal cell numbers, compared to respective uninhibited cultures (table 5.1, Fig. 5.6) . The number of neurons surviving in each condition is not statistically different; the apparent bigger neuronal loss in factor treated cultures stems from the increased neuronal numbers in the uninhibited, factor treated cultures compared to cultures grown without factors or LY294002 (Table 5.1), which suggests that LY294002 treatment can nullify the beneficial effects of both NT-3 and GDNF (Fig. 5.6).

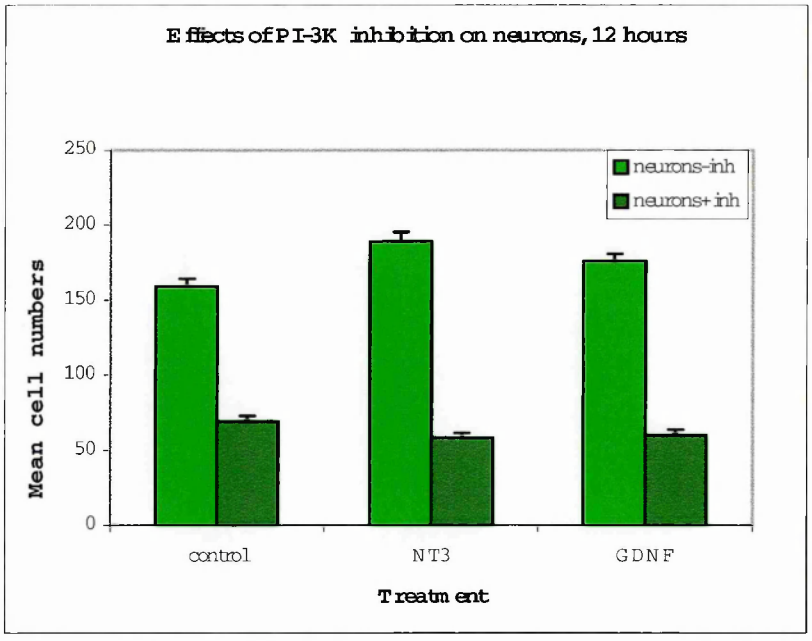


Figure 5.6 The effects of PI-3K inhibition on mean neuronal cell numbers after 12 hours incubation. Data are presented as a mean of 3 separate experiments, each treatment done in 4 replicas in each experiment. Error bars represent ± S.E.M.

Changes between inhibited and uninhibited cultures were even more pronounced at the 36 hour time point. LY294002 treatment caused a 83% reduction of glial cell numbers irrespective of factor treatment (Fig. 5.7).

Similarly, neuronal cell numbers in the inhibited cultures declined further than at 12 hours time point. PI-3K inhibition in the cultures grown without factors resulted in 75% reduction of neuronal numbers compared to uninhibited cultures (Fig. 5.8). LY294002 treatment of the cultures grown with NT-3 caused 84% reduction of neuronal numbers; a similar effect was observed in the inhibited cultures grown with GDNF, which displayed 80% neuronal decline compared to respective uninhibited controls (Fig. 5.8).

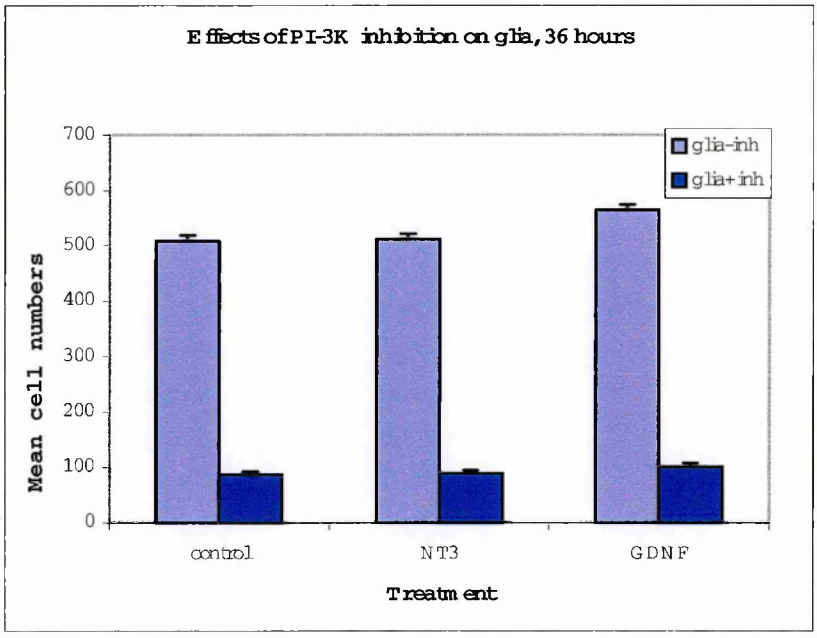


Figure 5.7 The effects of PI-3K inhibition on mean glial cell numbers after 36 hours incubation. Data are presented as a mean of 3 separate experiments, each treatment done in 4 replicas in each experiment. Error bars represent ± S.E.M.

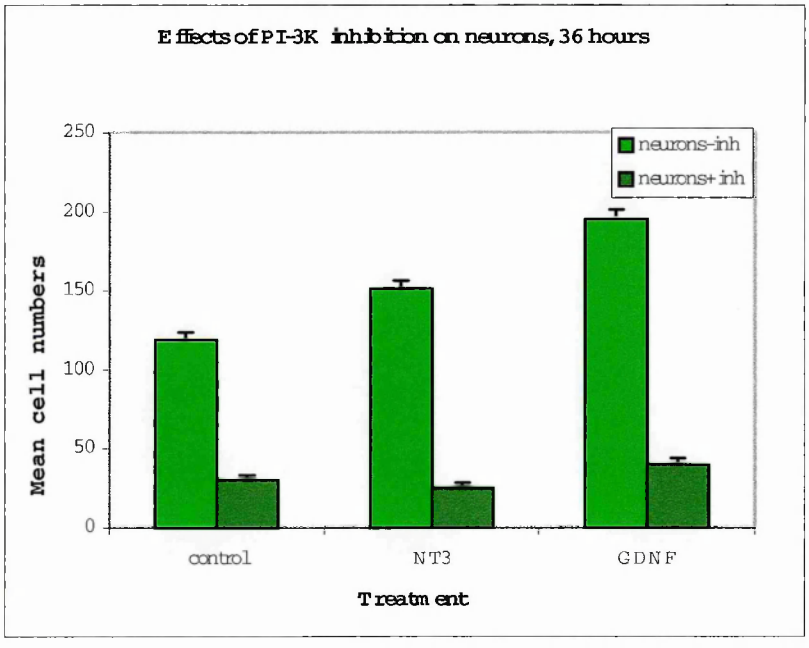


Figure 5.8 The effects of PI-3K inhibition on mean neuronal cell numbers after 36 hours incubation. Data are presented as a mean of 3 separate experiments, each treatment done in 4 replicas in each experiment. Error bars represent ± S.E.M.

The increased neuronal death rate observed in the inhibited cultures grown with NT-3 and GDNF compared to LY294002 treated cells grown without factors results from increased neuronal survival in factor treated, uninhibited cultures; the actual numbers of neurons surviving in LY294002 treated cultures at 36 hours are comparable in each condition tested. Such an effect was also observed in cultures grown for 12 hours (Table 5.1).

Comparison of the effects of PI-3K inhibition on neuronal and glial cell numbers between cultures grown for 12 and 36 hours reveals two facts. While glial cell numbers are affected to a lesser degree that neurons in cultures grown with inhibitor for 12 hours, continuous inhibition of PI-3K results in reduction of glial cell numbers comparable with that of neurons in cultures grown for 36 hours. Secondly, inhibition of PI-3K abolishes the beneficial effects of both NT-3 and GDNF on enteric neurons at both time points examined, reducing neuronal cell numbers in factor and inhibitor treated cultures to that of LY294002 treated control cultures.

5.4.2 Changes in the phosphorylation status of Akt, MAP and ERK kinases after NT-3 or combined NT-3/LY294002 treatment.

The results of the previous set of experiments have shown that PI-3K plays crucial role in the survival of enteric ganglion cells *in vitro*. The next set of experiments was performed in order to analyse the signalling pathways that are activated by neurotrophic factors in enteric ganglion cells in more detail.

Of the two neurotrophic factors tested, NT-3 exhibited a potential to protect enteric ganglion cells by initiating survival responses in the cells, while the effect of GDNF was primarily to increase cell numbers in the culture, perhaps by promoting proliferation. Based on these observations, further experiments concentrated on investigating the signalling cascades initiated by NT-3. It is accepted that NT-3/Trk binding initiates signalling along two major pathways, that of PI-3K/Akt and Raf/MEK/MAPK. Based on this information, the phosphorylation status of key molecules in these pathways, Akt, MEK and ERK was examined.

Due to the rapid nature of signal transduction cascades, lysates from cell cultures exposed to NT-3 were collected at the end of short intervals (0, 5, 20, and 35 minutes) after treatment and processed for western blotting. To address the question of the effect of PI-3K inhibition on NT-3 initiated signalling, designated cultures were incubated with LY294002 inhibitor at 300μM concentration for 30 minutes at 37°C, 2.5% CO_2 before NT-3 treatment to allow complete inhibition of PI-3K. Figure 5.9 shows the results of representative western blot immunolabelled with antibodies against phosphorylated forms of Akt, MEK and ERK proteins. Results obtained in separate experiments were comparable with the ones presented in the figure.

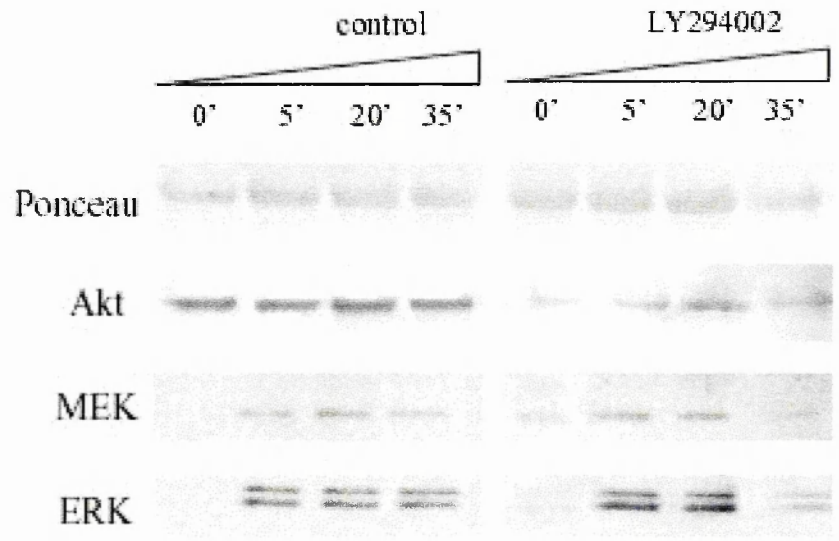


Figure 5.9 Western blotting against phosphorylated Akt, MEK and ERK proteins. Cell cultures established as described in materials and methods were grown for 12 hours, and subsequently exposed to 10ng/ml NT-3. Cells were collected at 0, 5, 20, 35 and 55 minutes after NT-3 treatment and processed for western blotting. LY294002 treatment was initiated 30 minutes before addition of NT-3 to allow inhibition of PI-3K. 5μg of protein extracts was loaded on to each gel. To ensure equal protein content on each lane, membranes after electro-blotting were incubated with ponceau red (top lane), and protein amounts were estimated using densitometry.

Phosphorylation levels of Akt remained constant at every time point examined, suggesting that exposure to NT-3 does not have an effect on the phosphorylation status of Akt. Considering the well-established role of the PI-3K/Akt pathway in survival responses in a number of different neuronal populations, it stands to reason that the PI-3K/Akt pathway is necessary for the survival of enteric ganglion cells *in vitro*, a conclusion supported by the data obtained in PI-3K inhibition experiments described in the previous section. In agreement with that hypothesis, LY294002 pretreatment reduced the amount of phosphorylated Akt. Although levels of phosphorylated Akt were lower in the LY294002 treated than in untreated cultures, densitometric analysis of the western blots of LY294002 treated cultures indicates a small increase of the levels of phosphorylated Akt following NT-3 treatment, reaching maximum 20 minutes after factor treatment, with a subsequent decline in the signal strength (Table 5.2, Fig. 5.10)

	control				LY294002			
	0'	5'	20'	35'	0'	5'	20'	35'
Akt	142±12	133±7	146±12	144±6	72±8	103±8	195±16	93±6
MEK	0	198±11	224±12	154±16	96±11	231±19	308±21	130±18
ERK	0	171±9	160±5	157±13	44±16	189±6	253±9	85±8

Table 5.2 Densitometric analysis of the effect of NT-3 and NT-3/LY294002 combined treatment on the phosphorylation status of Akt, MEK and ERK proteins. Data presented in the table are mean densitometric values gathered from three separate western blots.

Phosphorylation of MEK and ERK proteins in response to NT-3 stimulation showed a different pattern to that of Akt. At the point of NT-3 exposure, phosphorylated forms of MEK and ERK were barely detectable. However, just 5 minutes after NT-3 stimulation phosphorylated forms of both proteins give a strong signal, which continued until the last time point examined, 35 minutes after addition of NT-3 to the cultures. Pretreatment with LY294002 had no effect on the maximal signal intensity observed for both MEK and ERK proteins (table 5.2, compare densitometric values of MEK and ERK signal at 35 minutes time point with 20 minutes time point for LY294002 treated cultures). However, their phosphorylation pattern was altered. In both cases phosphorylation levels increased until 20 minutes after NT-3 exposure, with a subsequent decline of signal intensity, mimicking the pattern displayed by Akt. Also, a detectable levels of phosphorylated versions of both proteins were detected at the point of addition of NT-3, suggesting that it was independent of the actions of NT-3.

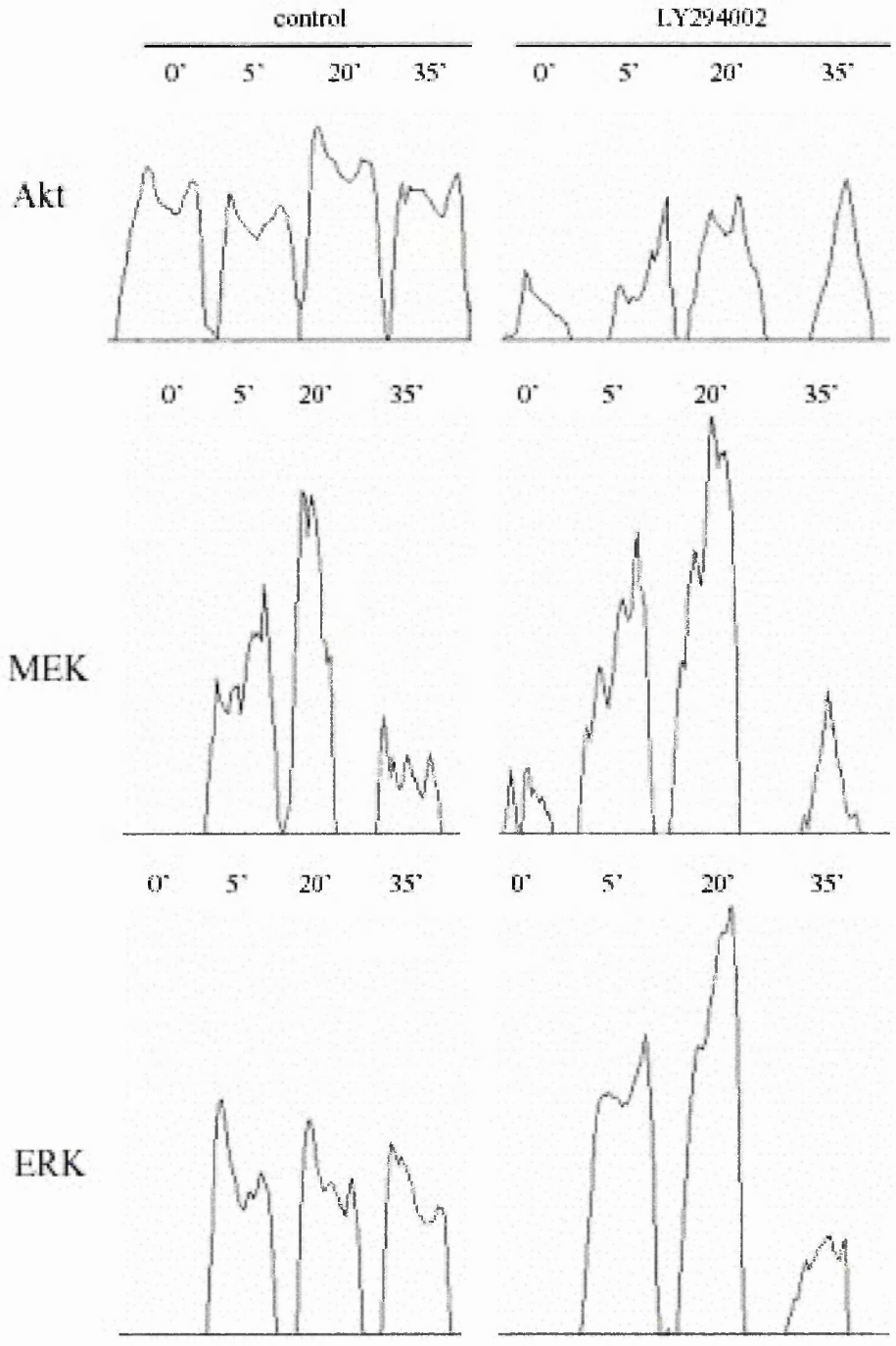


Figure 5.10 Densitometric graphs of western blots presented in figure 5.10. Densitometric peaks shown on the graphs correspond to the lanes on western blots for respective protein (Akt, MEK and ERK)

5.4.3 Changes in the protein levels of bcl-2 and c-fos initiated by NT-3 or combined NT-3/LY294002 treatment.

After investigating the effect of NT-3 and combined NT-3/LY294002 treatments on upstream signalling molecules in the PI-3K/Akt and MEK/MAPK pathways, the last set of experiments was performed to examine the changes in the status of downstream molecules of these pathways, bcl-2 and c-fos. Using the experimental design described previously (section 5.4.2), cell cultures treated with NT-3 with and without LY294002 were collected at 3 time points, 5, 20 and 35 minutes after factor treatment. Western blots were performed in order to investigate the amount of total bcl-2 and c-fos present in the lysates. Due to the slower rate of changes of the total amount of these proteins (15 minutes average half-life of c-fos), cell lysates were not prepared from the cells directly after NT-3 treatment (0 minutes time point). Representative western blots immunolabelled against total bcl-2 and c-fos proteins are shown in Figure 5.11, with densitometric graphs corresponding to the western blots presented in Figure 5.12. The results of densitometric analysis are summarized in Table 5.3.

Both c-fos and bcl-2 show little variation in protein levels at all time points in the NT-3 treated cultures, although levels of c-fos are slightly elevated after NT-3 treatment compared to bcl-2. Pretreatment with the PI-3K inhibitor LY294002 affects c-fos as well as bcl-2, causing elevation in levels of both proteins at every time point examined compared to controls. Moreover, western blots and densitometric data suggest that changes in the protein levels observed for both c-fos and bcl-2 in the inhibited cultures might parallel the pattern of changes in phosphorylation levels showed by Akt, MEK and ERK. After an initial increase in the protein content of both c-fos and bcl-2 seen at 5 and 20 minutes after NT-3 treatment,

compared to the respective times in control cultures, protein levels decreased between 20 and 35 minutes after exposure to NT-3 (Fig 5.11 and 5.12, table 5.3).

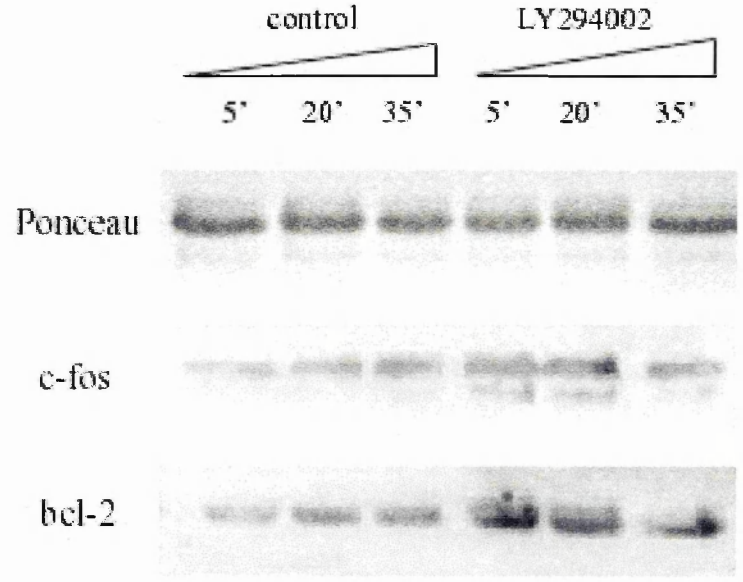


Figure 5.11 Western blotting against total bcl-2 and c-fos proteins. Cell cultures established as described in materials and methods were grown for 12 hours, and subsequently exposed to NT-3 at 10ng/ml concentration. Cells were collected at 5, 20, and 35 minutes after NT-3 treatment and processed for western blotting. LY294002 treatment was initiated 30 minutes before addition of NT-3 to allow inhibition of PI-3K. 5μg of protein extracts was loaded on to each gel. To ensure equal protein content on each lane, membranes after electro blotting were incubated with ponceau red (top lane), and protein amounts were estimated using densitometry

	control			LY294002		
	5'	20'	35'	5'	20'	35'
c-fos	125±18	110±10	130±10	284±9	247±11	195±11
bcl-2	102±10	96±4	116±4	219±13	268±14	143±15

Table 5.3 Densitometric analysis of the effect of NT-3 and NT-3/LY294002 combined treatment on the total amount of bcl-2 and c-fos proteins. Data presented in the table are mean densitometric values gathered from three separate western blots.

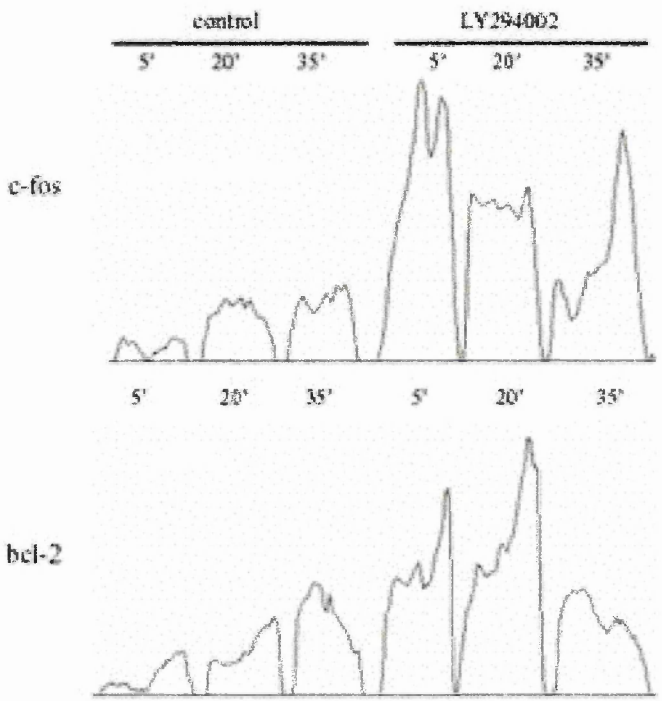


Figure 5.12 Densitometric graphs of western blots presented in figure 5.12. Densitometric peaks shown on the graphs correspond to the lanes on western blots for respective protein (c-fos and bcl-2)

5.5 Discussion

There is good evidence supporting the roles of the PI-3K/Akt and MEK/MAPK pathways in signalling initiated by activation of Trk receptors [Kaplan et al. 2000]. Numerous neuronal populations, including sympathetic and cerebellar neurons [Dudek et al. 1997, Virdee et al. 1999] have been shown to employ these pathways in survival responses resulting from activation of Trk receptors.

PI-3K, an enzyme known to be involved in many survival responses in neuronal populations of both CNS and ENS was a target of preliminary experiments. To examine the role of PI-3K in the survival of enteric ganglion cells, cell cultures were grown for 12 or 36 hours (with either NT-3 or GDNF) with or without LY294002, a specific inhibitor of PI-3K. As expected, inhibition of PI-3K resulted in a marked decrease in the numbers of both glial and neuronal cells present in the cultures after 12 hours, which implies that PI-3K is required for survival of both cell types *in vitro* and perhaps *in vivo*.

Irrespective of factor treatment, glial cell numbers were comparable among uninhibited cultures. Similarly, cultures treated with LY294002 displayed comparable glial cell losses in all treatments which suggests that NT-3 and GDNF have only minor, if any, effect on glial cell numbers. This observation in turn suggests that enteric glia are less dependent on exogenous trophic factor support then enteric neurons. The diminished glial numbers in the cultures treated with LY294002 also show a crucial role of PI-3K in the survival of enteric glial cells in *in vitro*, and most likely in *in vivo* situation.

LY294002 treatment caused a marked decrease in the numbers of neurons present in both cultures grown without factor support and grown with NT-3 and GDNF, compared to their respective, uninhibited controls. Cultures grown without factor support exhibited a 56% decrease in neuronal numbers compared to uninhibited control cultures. The neuronal loss was more extensive in the cultures grown with NT-3 or GDNF and LY294002, reaching an average of a 65% reduction.

The larger degree of neuronal loss in LY294002 treated cultures grown with NT-3 or GDNF compared to inhibited cultures grown without factors could be interpreted in two ways. The first possibility is that factor treatment increases neuronal death in cultures grown with LY294002. Alternatively, since the values of neuronal and glial cell loss were calculated as a ratio of the neuronal numbers in the respective controls to the neuronal numbers present in the inhibited cultures, higher death ratios could result from higher numbers of neurons present in the control cultures. This indeed seems to be the case, as at 12 hours time point cultures grown with either NT-3 or GDNF have higher numbers of neuronal cells compared to control (average 159 neurons per coverslip for controls, to 169 in NT-3 and 176 in GDNF treated cultures), which is also true at 36 hours.

Another important fact is the effect of PI-3K inhibition on the actions of NT-3 and GDNF on enteric ganglion cells. The average number of neurons per area counted in control cultures grown without inhibitor at 12 hours is 159, NT-3 increases that number to 189, while GDNF increases neuronal numbers to 176 (see Table 5.1). Addition of LY294002 to untreated, NT-3 or GDNF treated cultures decreases these numbers to 69, 58 and 60, respectively. The effect of PI-3K inhibition is similar at 36 hours, where cultures grown without factor support have average 119 neurons per coverslip, NT-3 treated cultures 151 and GDNF treated cells 195

neurons, which in case of GDNF exceeds the numbers of neurons at 12 hours. Inhibition of PI-3K decreases these numbers to 30, 25 and 40, respectively. Both at 12 and 36 hours, inhibition of PI-3K blocks the effects of both factors bringing the number of neurons in each treatment to comparable level. This observation suggests that PI-3K is an important step in the signalling cascades initiated by both factors, since its inhibition nullifies the effects of both NT-3 and GDNF on enteric neurons, bringing cell numbers down to the levels observed in control.

The changes observed in LY294002 treated cultures were more pronounced after 36 hours. Both glial and neuronal populations displayed dramatic decreases in each treatment compared to controls. Interestingly, while initially (after 12 hours growth) inhibition of PI-3K affected glia to lesser degree than neurons, at 36 hours glia and neurons were affected in similar way, displaying comparable degree of cell death. Glial cell numbers decreased by 50% in all treatments compared to uninhibited controls after 12 hours, after 36 hours glial cell numbers decreased by 83%, compared to 65% of neuronal loss at 12 hours and 84% at 36 hours in the LY294002 treated cells grown with factors.

Taken together, the data discussed above imply that (i) glial cells are relatively independent of neurotrophic factor support for survival, probably due to generation of other endogenous trophic factors. (ii) Survival of enteric glia is dependent on signalling pathways involving PI-3K, a hypothesis supported by greatly increased death rate of glia in cultures grown for both 12 and 36 hours with LY294002.

Based on the fact that even at 36 hours some cells are still present in the LY294002 treated cultures one more conclusion could be made. Enteric ganglion cells, or at least some populations of these cells, seem to be able to partially compensate for inhibition of PI-3K,

perhaps by bypassing PI-3K and activating Akt by a different pathway or by ongoing activation of another pathway that is unaffected by PI-3K inhibition, which allows these cells to survive blockage of the primary, PI-3K/Akt survival pathway. Such a scenario is possible, considering that it has been proposed that Akt could be modulated by pathways other than the Ras/PI-3K pathway.

The next step was to investigate the molecules involved in signalling cascades initiated by NT-3/Trk receptor, as well as changes in signalling events caused by inhibition of PI-3K. Data obtained in previous chapters indicated that while NT-3 has the ability to promote survival responses in cultured enteric ganglion cells, the effect of GDNF is mainly to increase cell numbers in the cultures, possibly by inducing proliferation of precursor cells. Considering these observations, NT-3 seemed to have more potential as a possible factor in therapies designed to prevent or reduce neuronal losses associated with oxidative stress in adults than GDNF, since it is unlikely that adult gut might contain sufficient cells able to respond to proliferative signals. For that reason, only NT-3/Trk initiated signalling cascades were examined in this series of experiments.

Due to rapid changes in the phosphorylation status of signalling molecules after signal initiation, a different experimental design was used than previously. Cell lysates were prepared from cell cultures in short intervals after exposure to NT-3 with and without LY294002, up to maximum of 35 minutes after the beginning of the experiment. The results of these experiments show constant levels of Akt phosphorylation across every time point examined, including the time of NT-3 addition, independent of NT-3 treatment. These results suggest that the PI-3K/Akt pathway is crucial for survival of enteric ganglion cells, and as such is likely to

be constitutively active especially in *in vitro* conditions, a conclusion supported by the results of the previous experiments.

Not surprisingly, LY294002 pretreatment reduced the amount of phosphorylated Akt present in the cells, as well as the time frame of Akt phosphorylation. Levels of phosphorylated Akt remained lower in inhibited cultures compared to controls, however, a slight increase in the strength of western blot signal occurred between 0 and 20 minutes after NT-3 treatment, confirmed by densitometric analysis of western blots. Levels of phosphorylated Akt seemed to decline after 20 minutes, which continued until the last time point examined. This observation suggests that NT-3 signalling is able to affect phosphorylation of Akt, an effect that could be masked by constitutively high levels of phosphorylated Akt present in uninhibited cultures. The increase of phosphorylated Akt in the inhibited cultures most likely is not linked with PI-3K which could imply that that Akt phosphorylation might be regulated not only by PI-3K, but also by another pathway, independent of PI-3K.

Investigation of MEK and ERK proteins, participating in the second major signalling pathway present in the neurons, revealed a different behaviour than that observed for Akt. Phosphorylated forms of both proteins are undetectable at the moment of NT-3 treatment, however they undergo rapid phosphorylation as a result of activation of the Trk receptor, and give a strong signal beginning from 5 minutes after NT-3 treatment, continuing until the last time point examined. A double band was observed in case of ERK corresponds to two isoforms of ERK protein. A lack of clear differences in the intensity between these bands suggests that NT-3 stimulation affects phosphorylation of both isoforms to the same degree.

The rise in the amount of phosphorylated forms of MEK and ERK proteins following NT-3 stimulation suggests that the MEK/MAPK pathway, in contrast to the PI-3K/Akt pathway, is not constitutively active in the enteric ganglion cells, and instead is activated in response to exogenous trophic factor stimulation. Although not performed in this study, use of specific inhibitors of MEK kinase should confirm this hypothesis.

One explanation of this result could be that while the PI-3K pathway is mainly involved in cell survival (as discussed in section 5.1.2), primary actions of the MEK/MAPK pathway are induction of differentiation or cell proliferation [Grewal et al. 1999]. In the light of these facts it is feasible to assume that the effects of both NT-3 treatment, such as neurite outgrowth observed in the cultures of enteric ganglion cells, as well as increases in neuronal numbers observed in cultures grown with GDNF are at least partially dependent on MEK/MAPK signalling. These actions of the MEK/MAPK pathway can also explain induction of signalling along this pathway upon NT-3 stimulation, and lack of MEK/MAPK phosphorylation in the absence of trophic factor, since the activation of this pathway seems to depend on extracellular stimuli inducing differentiation responses, which are not required for neuronal survival.

Inhibition of PI-3K changed the time course of phosphorylation of both MEK and ERK. In contrast to controls, LY294002 treated cells show small, but detectable levels of phosphorylated forms of these proteins at the point of NT-3 addition with subsequent raise of signal strength which peaks 20 minutes after NT-3 treatment and declines afterwards. Since the maximum intensity of the bands for both MEK and ERK obtained in western blots performed on lysates from LY294002 treated cultures is comparable to uninhibited controls, it stands to reason that phosphorylation of MEK and ERK is not dependent on PI-3K, which remains in accordance with generally accepted view.

The small increase in the amount of phosphorylated MEK and ERK seen at the point of NT-3 stimulation in the inhibited cultures might indicate that cells devoid of PI-3K activity initiate other pathways promoting cell survival, for example MEK/MAPK and perhaps some others. If true, it might explain small levels of phosphorylated MEK and ERK observed at the point of NT-3 exposure, considering that inhibitor of PI-3K was added 30 minutes before addition of NT-3, which allows the cells to initiate alternative survival signalling before addition of NT-3 to the cultures. The initial increase of phosphorylation levels of MEK and ERK seen in the cultures treated with LY294002 and the subsequent decrease beginning 20 minutes after NT-3 exposure could be explained in several ways. Perhaps the most likely explanation is that NT-3 initiated signalling requires interplay between PI-3K/Akt and MEK/MAPK pathways for continuous activity of both pathways. In the situation where PI-3K signalling is removed, the MEK/MAPK pathway does not receive feedback and its activity decreases, which could explain drop in the phosphorylation levels of both MEK and ERK after 20 minutes after NT-3 treatment.

Interestingly, the time course of changes in the levels of phosphorylated MEK and ERK in the inhibited cultures are similar to these observed in case of Akt. This suggests interaction between PI-3K/Akt and MEK/MAPK pathways, perhaps even existence of a feedback between Akt and MEK/MAPK. However, more data are required to validate this hypothesis.

The last set of experiment was focused on the downstream effectors of both PI-3K/Akt and MEK/MAPK pathways, namely bcl-2 and c-fos proteins. Using western blots, the levels of total bcl-2 and c-fos were examined in uninhibited, as well as LY294002 treated cultures. Both proteins are detectable at every time point examined, and their levels remain constant.

Addition of LY294002 results in marked increase of both bcl-2 and c-fos protein content in the cell lysates, compared to uninhibited controls. Similarly to Akt, MEK and ERK, levels of bcl-2 and c-fos in the inhibited cultures decrease after 20 minutes, which parallels the decrease in the phosphorylation levels observed for Akt, MEK and ERK. Since both bcl-2 and c-fos are downstream effectors of many pathways, including both the PI-3K/Akt and MEK/MAPK pathways, it stands to reason that changes in the activity of these pathways might be accompanied by similar changes of effector molecules, such as bcl-2 and c-fos.

Taken together the results obtained in this study confirm the role of PI-3K/Akt as an important survival pathway in both enteric neurons and glial cells. Inhibition of PI-3K caused dramatic decrease in the numbers of cells present in the cultures. Such an effect is most likely associated with decrease in the phosphorylation of Akt. Since Akt is known to repress several pro-apoptotic proteins, decrease of Akt activity is likely to cause an activation of these proteins, resulting in the initiation of the cell death program, which is the most likely the reason for the observed decrease in the numbers of neurons and glial cells. The data confirming this assumption come from western blots, which showed constant, high levels of Akt phosphorylation in the uninhibited cultures, supporting the role of PI-3K/Akt pathway in the survival of enteric ganglion cells. Although the data gathered in this study describe an *in vitro* situation, it is feasible to assume that PI-3K signalling plays equally important role *in vivo*.

To summarize, the experiments described in this chapter provide data confirming the role of PI-3K/Akt signalling pathway in survival of enteric ganglion cells *in vitro*. Furthermore, a role of MEK/MAPK pathway was established as one of the signalling pathways underlying the effects of NT-3, and possibly GDNF on enteric ganglion cells in culture.

Chapter 6

General discussion

There is considerable evidence documenting changes occurring in the gut of aged animals. One of the hallmark features of aging in the gut is the loss of enteric neurons [Gabella et al. 1989, Santer et al. 1988], which is likely to contribute to the numerous disorders of gastrointestinal tract observed in elderly. Although there are no practical means of reversing the effects of aging in humans available to date, caloric restriction has been consistently shown to prevent at least some age-associated changes in every animal model examined, from the nematode *C. elegans* to rats.

A considerable amount of research has been focused on finding methods to counteract age-related changes in humans. Due to its beneficial effects in animal models, caloric restriction has drawn some attention as a promising avenue of research. The results of several studies demonstrate that caloric restriction alters the expression of a broad spectrum of proteins, ranging from metabolic enzymes to free radical scavenging enzymes, as well as reducing generation of free radicals due to the more efficient usage of available energy sources [Hagopian et al. 2002]. It has been demonstrated that in calorically restricted animals neurotrophic factor treatment can further reduce free radical production in the gut *in vitro* [Kim et al. 1997, T. Cowen, personal communication]. Based on those observations, current study has been undertaken in order to find out the possible protective role played by NT-3 and GDNF in the survival of enteric ganglion cells, especially enteric neurons, *in vitro*.

The protective effects of neurotrophic factors against H_2O_2 induced death.

The first set of experiments, described in chapter 3, investigated the effects of NT-3 and GDNF on the cultures of enteric ganglion cells exposed to oxidative stress induced by H_2O_2. Not surprisingly, H_2O_2 treatment reduced cell numbers in the cultures. Simultaneous addition of NT-3 had a slight protective effect on cell viability, measured using MTS assay, while no such effect was detected for GDNF. In a separate series of experiments counts of Hoechst/propidium iodide stained cultures exposed to H_2O_2 in conjunction with the factor treatment showed a significant reduction in the number of dead cells present in the H_2O_2 treated cultures that received NT-3 compared to controls, which reinforced the data obtained in MTS assays. These data remain in agreement with the results of other studies showing beneficial effects of neurotrophins on neuronal cells exposed to oxidative stress [Heaton et al. 2000, Miyazaki et al. 1999].

The difference in the protective effect of NT-3 observed using MTS assay and Hoechst/Propidium iodide staining could stem from different sensitivity of the two methods. While MTS offers a fast way of examining the changes of cell viability, it is able to detect only gross changes occurring in the cultures, while counting cells labelled with Hoechst/Propidium iodide is a much slower, but also more accurate method. On the other hand, GDNF was not able to counteract cell death in H_2O_2 treated cultures, instead increasing both neuronal and glial cell numbers in the control cultures. Interestingly, H_2O_2 treatment abrogated this effect. The explanation of this phenomenon could be that cell death resulting from H_2O_2 exposure, or perhaps signalling pathways in the cells activated by exposure to H_2O_2 [Kim et al. 1997, Cabalerro et al. 1997] interfered with signalling required for GDNF to exert its effects on the cells.

Similarly, cultures exposed to combined NT-3 and GDNF treatment without H_2O_2 behaved in a manner comparable to control cultures, implying that NT-3 and GDNF, when applied simultaneously, can nullify each other's effects. Since in the experimental model used in this study NT-3 treatment induced a differentiation response in the cultures, such as neurite outgrowth, while GDNF induced proliferation it is possible that activation of these conflicting pathways, that is differentiation and proliferation at the same time was the cause of a reduction of the effect of both NT-3 or GDNF in the combined treatment. The intracellular mechanism responsible for this effect might include competition for signalling molecules between pathways conveying signals initiated by NT-3 and GDNF, since both factors employ similar signalling events to convey their effects [Kaplan et al. 1997]. Another possibility could be that end effects of pathways induced by GDNF and NT-3 are mutually exclusive, in which case responses initiated by both factors would nullify each other.

The effects of neurotrophic factors on the expression of catalase and SOD Cu/Zn.

The results discussed in chapter 3 raised another question, that is of the mechanism responsible for the protective effect of NT-3. One possible explanation, supported by the results obtained by other groups, is that NT-3 treatment might increase the expression or activity of antioxidant enzymes present in the cultures, as has been documented for other neurotrophic factors [Chao et al. 1999], thereby reducing the amount of oxidative damage to the cells and decreasing the amount of cell death. To test this hypothesis, protein levels of two of the antioxidant enzymes present in enteric ganglion cells, catalase and SOD Cu/Zn, were examined using western blotting as described in the results of chapter 4. The results indicated no significant changes in the levels of these enzymes after 12 hours incubation with either

NT-3 or GDNF. 36 hours treatment with NT-3 resulted in a small decrease in the levels of SOD Cu/Zn. Similar treatment with GDNF caused significant increase in the levels of both catalase and SOD Cu/Zn, compared to controls.

At first sight this result seems somewhat contradictory to the protective effects of NT-3 observed earlier. However, one has to bear in mind the difference in the experimental design used in the two sets of experiments. While preliminary experiments focused on the short term protective effects of NT-3 and GDNF subsequent experiments concentrated on the longer-term effect of factor treatment on the expression of antioxidant enzymes. This difference might explain the discrepancy between the results of the two sets of experiments. However, since NT-3 treatment does not have significant effects on the levels of the two antioxidant enzymes tested, that is catalase and SOD Cu/Zn, it seems more feasible to assume that the protective effects of NT-3 are associated either with increased protein levels of other antioxidant enzymes, for example SOD Mn, or increased *activity* of antioxidant enzymes present in the cells. Other possible mechanisms by which NT-3 might convey its protective effects are discussed later.

Since prolonged GDNF treatment increased protein levels of both the antioxidant enzymes tested, a possibility arises that GDNF treatment might offer protection against oxidative insults upon prolonged exposure. If true, such an effect could make GDNF an attractive target of therapies focused on preventing neuronal losses resulting from oxidative stress. However, more data are required to validate this hypothesis.

To address the question of possible differential expression of the two enzymes by neurons and glial cells as well as the contribution of both cell types to the total cell numbers present in the

cultures, cells grown for 12 and 36 hours were fixed and immunolabelled using the antisera raised against catalase and SOD Cu/Zn or PGP 9.5, respectively. Catalase and SOD Cu/Zn immunostaining shows strong, clear staining of neurons and weaker staining of glial cells, with staining intensity of both cell types comparable between cultures stained at 12 and 36 hours. This observation suggests that neurons might contain higher levels of both enzymes than glial cells, which stands to reason if one considers higher metabolic rate of neuronal cells compared to glia, and resulting requirement for more robust defence systems.

Cell counts of PGP 9.5 stained cultures revealed that while the neuron to glial cell ratio decreased in both control and NT-3 treated cultures at the 36 hours time point, it remained almost constant in the GDNF treated cultures. This fact, together with the increase in both neuronal and glial cell numbers that seems to take place between 12 and 36 hours suggests that GDNF is able to induce proliferation in the cultures of enteric ganglion cells used in this study, which confirms the results of cell counts performed on Hoechst/propidium iodide stained cultures. Taken together, those results suggest that both neurons and glial cells contribute to the increase in the levels of SOD Cu/Zn and catalase observed in the cultures grown with GDNF for 36 hours. The fact that increases in the levels of catalase and SOD Cu/Zn were only seen after prolonged incubation with GDNF might explain the lack of protective effect of GDNF observed previously in the cultures exposed to H_2O_2 after 12 hours.

Taken together, the results from investigation of the effects of NT-3 and GDNF treatment on the levels of catalase and SOD Cu/Zn argues against the hypothesis that the protective effect of NT-3 on H_2O_2 treated cultures observed earlier is due to NT-3 dependent increase of the levels of these enzymes, although it cannot be ruled out that NT-3 is able to affect other antioxidant enzymes, such as SOD Mn or GPx, which might at least partially contribute to the

protective effect of NT-3. Alternatively, NT-3 treatment could affect the activity of antioxidant enzymes instead of increasing their protein levels. Such an effect might explain the protective effects of NT-3 and the lack of its effect on the levels of antioxidant enzymes present in the cultures.

The effects of neurotrophic factors on signalling pathways in enteric ganglion cells.

Another possible explanation of the protective effect of NT-3 is that the intracellular signalling events initiated by NT-3 treatment are able to override the death program initiated by H_2O_2. To address the question of a possible molecular mechanisms employed by NT-3 to convey its protective effects, intracellular signalling cascades initiated by NT-3 have been examined, as described in chapter 5. The first step was to assess the role of PI-3K, an enzyme involved in survival responses in numerous neuronal populations [Virdee et al. 1999, Kaplan et al. 2000] in the NT-3 induced signalling in enteric ganglion cells *in vitro*. A specific inhibitor of PI-3K, LY294002, was used on cultures grown with NT-3 or GDNF for 12 or 36 hours, and the effects of PI-3K inhibition were assessed by cell counts of PGP 9.5 stained cultures.

The results clearly indicated the importance of PI-3K signalling to the survival of enteric ganglion cells *in vitro*. Inhibited cultures, regardless of factor treatment, showed extensive cell loss after 12 hours, which increased at 36 hours. Both neuronal and glial cell populations were affected by LY294002 treatment. At the 12 hour time point, glial cells exhibited smaller losses compared to neurons, however between 12 and 36 hours the death rate of glial cells increased, resulting in similar loss of glial and neuronal cells at 36 hours.

Although neither NT-3 nor GDNF treatment were able to rescue the cells from the effects of PI-3K inhibition, neuronal death rate was slightly different between NT-3 and GDNF treated cultures while glial cells were affected in the same manner in each condition. This observation suggests that glial cells might be less dependent on neurotrophic factor signalling than neurons. This effect could be caused by the production of endogenous trophic factor support by glial cells. However, it seems that PI-3K signalling is required for survival of glial cells as well as neurons, as shown by similar degree of cell death in both populations after 36 hours exposure to PI-3K inhibitor.

Having established that PI-3K is necessary for the survival of enteric ganglion cells, the signalling events occurring in the enteric ganglion cells after NT-3 stimulation were investigated. It is believed that in neurons signalling initiated by binding of neurotrophins to their cognate receptors follows two major pathways, that of Ras/PI-3K/Akt and MEK/MAPK pathway [see Kaplan et al. 2000]. Based on this fact three proteins participating in propagation of the signal along these pathways were chosen, that is Akt, MEK and ERK, and their phosphorylation status corresponding with activation of these proteins was examined using western blotting.

Steady, easily detectable levels of phosphorylated Akt were detected at every time point in the cultures not treated with LY294002, beginning from the point of NT-3 stimulation to 35 minutes after the beginning of the experiment. Considering the fact that Akt is a main downstream target of PI-3K signalling, responsible for inhibition of several pro-apoptotic proteins [Dudek et al. 1997], such constant activation of Akt is not surprising, especially taken together with the detrimental effects of PI-3K inhibition on the survival of enteric ganglion cells. This result implies that ongoing activity of Akt is necessary for survival of enteric

ganglion cells *in vitro*, and quite likely also *in vivo* and that at least in the *in vitro* situation, activation of Akt is independent of signalling initiated by exogenous trophic factors.

The results obtained for MEK and ERK proteins were different to those of Akt. At the time of NT-3 stimulation the levels of phosphorylated forms of both proteins were not detectable in the cultures untreated with PI-3K inhibitor, however they rose just 5 minutes after NT-3 treatment and remained consistently high until the last time point examined. Considering that there are data suggesting that the MEK/MAPK pathway is involved in differentiation responses initiated by neurotrophin signalling in neuronal cells [Traverse et al. 1992], one might expect that NT-3 treatment would induce signalling along the MEK/MAPK pathway because morphological changes were induced by NT-3 stimulation in the cultures of enteric ganglia observed in this study.

Two downstream effector proteins activated by both the PI-3K/Akt and MEK/MAPK pathways were also studied. However, neither total protein levels of bcl-2 nor c-fos protein changed in the uninhibited cultures in the course of experiment, although they were readily detectable at every time point examined. In case of c-fos, this result most likely reflects the ongoing signalling events taking place in the cells, and remains in accordance with well-documented role of c-fos as a transcription factor activated in response to a range of stimuli both *in vivo* and *in vitro* [Sainz et al. 1998, Seternes et al. 1998]. The presence of bcl-2 protein might be related to its function as an anti-apoptotic protein [Seyfried et al. 2003], since it is likely that in the *in vitro* cultures where conditions are different from *in vivo* situation increased levels of bcl-2 might protect the cells from apoptosis.

The levels of Akt, MEK, ERK, bcl-2 and c-fos differed significantly in the NT-3 treated cultures pretreated with LY294002 inhibitor. Levels of phosphorylated Akt were markedly reduced compared to uninhibited controls. Nevertheless, densitometric analysis of western blot membranes showed a small increase in the amount of phosphorylated Akt 20 minutes after NT-3 addition, after which a decrease of signal occurred.

Although inhibition of PI-3K did not affect the maximum degree of phosphorylation of MEK and ERK, the time course of phosphorylation of both proteins was similar to that of Akt. Phosphorylated forms of both proteins were detected at the point of NT-3 treatment, indicating that some signalling events took place before NT-3 stimulation. It could be hypothesized that, since the primary survival pathway has been inactivated by PI-3K inhibition, the MEK/MAPK pathway might have been activated in order to compensate for the absence of PI-3K survival signalling. On the other hand, changes in the phosphorylation levels of MEK and ERK could also be related to the possible interplay between PI-3K/Akt and MEK/MAPK pathways.

Analysis of western blots indicated that after addition of NT-3, the levels of phosphorylated MEK and ERK increased in a manner similar to that observed for Akt, with a subsequent decrease in the amount of phosphorylated forms of both MEK and ERK beginning 20 minutes after NT-3 treatment. This similar time course of phosphorylation of Akt, MEK and ERK after NT-3 stimulation suggests existence of interplay between PI-3K/Akt and MEK/MAPK pathways. Interestingly, changes in bcl-2 and c-fos levels followed the same pattern. In the inhibited cultures levels of both proteins are increased compared to controls, and both bcl-2 and c-fos levels showed a decrease 20 minutes after NT-3 stimulation. One possible explanation of this behaviour could be that since the signalling along the PI-3K/Akt and MEK/MAPK pathways seemed to decrease in the inhibited cultures at the same time, that is

20 minutes after NT-3 application, which might be closely followed by their downstream effectors such as c-fos and bcl-2.

Taken together, these results indicate that both the PI-3K/Akt and MEK/MAPK pathways are activated by NT-3 binding to its receptor, even though such activation is masked in the case of the PI-3K/Akt pathway due to intrinsic high levels of phosphorylation of Akt which are independent of exogenous NT-3 signalling.

Inhibition of PI-3K provides an interesting insight into the actions of both pathways. Akt is directly affected by PI-3K inhibition, as a downstream target of PI-3K. Surprisingly, inhibition of PI-3K also affects the MEK/MAPK pathway, as shown by the change of time course of phosphorylation of both MEK and ERK upon PI-3K inhibition. Considering the decrease of signal strength from phosphorylated forms of MEK and ERK observed in the cultures pretreated with LY294002, mimicking the pattern displayed by Akt, it seems possible that there is some degree of interaction between the PI-3K/Akt and MEK/MAPK pathways. Another fact supports this theory. PI-3K signalling has been blocked in LY294002 treated cultures, yet Akt showed an increase of phosphorylation in response to NT-3 treatment. It could be argued that PI-3K has not been completely blocked by LY294002, and that Akt has been phosphorylated by residual activity of PI-3K. However, this does not seem likely, as high concentration of LY294002 used in the experiments, as well as the time between addition of an inhibitor and factor treatment should be able to block all residual PI-3K activity irreversibly. Another possible explanation is that a rise in Akt phosphorylation might have been caused by interaction with components of MEK/MAPK pathway [Zimmermann et al. 1999].

Based on these results, a possible model of NT-3 initiated signalling and its effects on the cultures of enteric ganglion cells is shown in Figure 6.1.

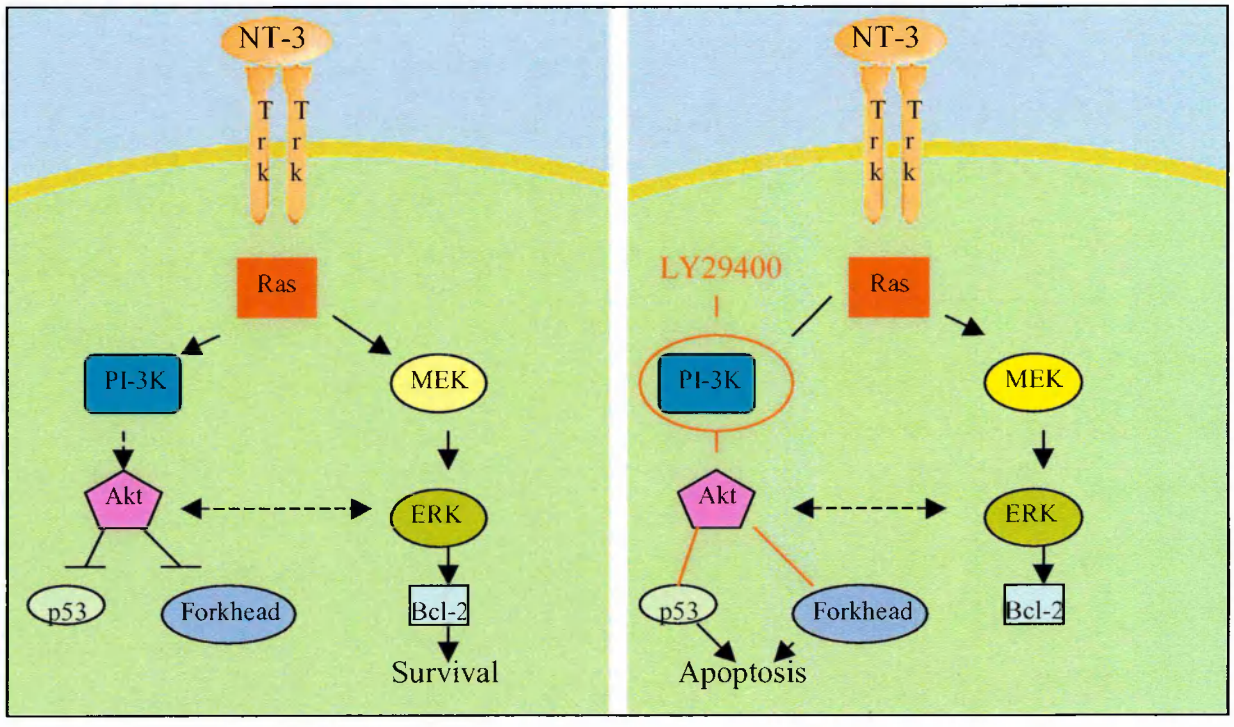


Fig. 6.1 Putative model of NT-3 induced signalling pathways (A) and the effects of PI-3K inhibition (B) in the enteric ganglion cells. Dashed line between Akt and ERK marks possible interaction between these proteins, suggesting the existence of a regulatory feedback between PI-3K/Akt and MEK/MAPK pathways. For more details see text.

NT-3, upon binding to its receptor initiates its phosphorylation. Subsequently, signal is transduced along the PI-3K/Akt and MEK/MAPK pathways, resulting in promotion of cell survival by reinforcing Akt stimulation and promoting differentiation responses as a result of MEK/MAPK signalling. LY294002 inhibition abrogates PI-3K signalling, resulting in deactivation of Akt, which in turn leads to derepression of pro-apoptotic molecules resulting in cell death. The putative interaction between Akt and MEK/MAPK pathway has also been

Although signalling cascades initiated by GDNF were not examined, it seems likely that GDNF signalling is not able to counteract the inhibition of PI-3K. This hypothesis is supported by the cell counts of cultures grown with combined GDNF and LY294002 treatment, where GDNF was not able to rescue either glia or neurons from cell death resulting from PI-3K inhibition.

Conclusions

The research undertaken in this study was based on the observation that caloric restriction increases neuronal survival, and also on the results obtained by Thrasivolou et. al. showing decreased amount of free radicals detected in the gut of calorically restricted animals, resulting from NT-3 treatment *in vitro* [Thrasivolou et al. 2000]. The goal of work described in previous chapters was to answer the question whether NT-3 and GDNF can protect enteric ganglion cells *in vitro* from oxidative stress, and also what mechanisms are responsible for the gross effects of these factors. Due to the high complexity of these processes, many interesting issues have not been addressed; some of them are discussed below.

The protective effects of NT-3 on enteric ganglion cells described above may well be due to the an end effect of several intracellular processes not examined here. Although decreased generation of free radicals does not seem likely due to short time frame of these experiments, which precludes synthesis of new proteins required for increased efficiency of the mitochondrial electron transport chain, other mechanisms can contribute to the observed protective effects of NT-3 and decreased amount of free radicals detected after NT-3 treatment by Thrasivolou et. al.

Several groups have reported increase in the activity of antioxidant enzymes after trophic factor treatment [Chao et al. 1999, Spina et al. 1992]. Such an effect might not be dependent on *de novo* synthesis of these enzymes, but instead on altering efficiency of existing antioxidant enzymes present in the cells. If true, such action of neurotrophic factors would not require long periods of time to take effect, which fits in with the results of this study. Another possibility is that NT-3 treatment might affect expression of antioxidant enzymes other than the ones tested in this study. Mitochondrial SOD or GPx are likely candidates. A novel observation of this study is the ability of GDNF to increase levels of catalase and superoxide dismutase in enteric ganglion cells after prolonged incubation. Such an effect remains in agreement with results of other groups, showing comparable increases not only in catalase and SOD Cu/Zn, but also GPx levels *in vivo* [Chao et al. 1999]. Simultaneous increase in the levels of all these proteins is an important fact, since increase in the levels of SOD Cu/Zn without parallel increase in catalase/GPx levels is thought to promote oxidative stress [Huang et al. 1992].

The impact of NT-3 on intracellular signalling cascades promoting cell survival rather than cell death could also contribute to the protective effect of NT-3. It is known that in case of some cell types increased expression of transcription factor CREB (cAMP response element binding protein) is sufficient to promote cell survival [Riccio et al. 1999]. This protein is a likely target of neurotrophic factor induced survival responses, as both the PI-3K/Akt and MEK/MAPK pathways are able to activate this protein [Bonni et al. 1999, Du et al. 1998]. In case of MEK/MAPK pathway activation of CREB is somewhat indirect, as it requires activation of Rsk proteins, a downstream effectors of ERK that in turn activate CREB [Bonni

et al. 1999]. Rsk proteins are able to promote survival in yet another way, by inhibition of Bad.

All these proteins offer attractive targets of therapies designed to prevent neuronal losses due to oxidative stress. The question of practical application of these data remains, however. To date, gene therapies or grafting modified cells are a popular approach to counteract adverse effects of oxidative stress and other conditions resulting in neuronal loss [Dumas et al. 2001, Naoi et al. 2001]. Both these methods use neurotrophic factors as means of protecting the cells. Although not yet feasible for practical trials, it is possible that therapies targeting particular genes, for example those of antioxidant enzymes or survival promoting proteins, like CREB or bcl-2 family will become available in close future.

Another approach to counteracting deleterious effects of oxidative stress are chemical compounds with free radical scavenging properties, or alternatively chemicals that mimic actions of certain antioxidant enzymes removing free radical species from the cells.

In both gene manipulation techniques and the more direct approach of chemical compounds with antioxidant properties, delivery to specific cell types is likely to pose a major obstacle. However, viral vectors and targeted liposomes are promising tools, able to deliver DNA/RNA or proteins and chemicals to specific cell types. Although in the stage of tests, these delivery systems show great promise as useful tools in therapies preventing neuronal loss.

In conclusion, the data gathered in this study supports the role of NT-3 as a survival factor for enteric neurons and glial cells, as shown by increased cell numbers in the cultures exposed to H_2O_2 with NT-3 treatment. The protective effect of NT-3 does not depend on increased levels

of catalase or SOD Cu/Zn and is detectable within 6 hours after NT-3 stimulation, which suggests that NT-3 treatment might offer an effective short term means of counteracting neuronal cell death caused by oxidative stress *in vitro*.

GDNF has not been able to rescue enteric ganglion cells from death induced oxidative damage after 6 hours exposure, instead inducing cell proliferation. However, GDNF dependent increases in the levels of catalase and SOD Cu/Zn after 36 hour exposure suggest that prolonged GDNF treatment might offer an effective protection against free radical damage. Such an effect makes GDNF a promising target of a long-term therapies aimed at preventing oxidative damage, and associated with it neuronal loss.

Future directions

The results of the experiments conducted in this study revealed several interesting facts concerning both the effects of neurotrophic factors on cellular antioxidant defences as well as signalling pathways responsible for protective actions of NT-3 and GDNF.

Nevertheless, many interesting issues remain to be addressed.

For example, in the culture model employed in this study both glial and neuronal cells are present. This fact could influence the behaviour of the two types of cells in response to exogenous factor stimulation or stress conditions, due to the endogenous trophic factor production and secretion by glial cells. Although separation techniques, for example gradient centrifugation would be hard to employ here due to small amount of cells obtained in preparations, seeding cells at lower density than used in this work might partially solve the problem of the interaction between glia and neurons, perhaps offering more accurate data.

Another issue is the way of inducing oxidative stress used in the experiments. Although H_2O_2 is a popular means of inducing oxidative stress, adding it to culture medium allows it to react with cellular components present on the cell surface before H_2O_2 penetrates into the cell. This might disturb the picture obtained from the experiments for two reasons. First, the oxidative stress will be more severe close to the point where H_2O_2 was added to the culture, and second, since H_2O_2 reacts with all cellular components as it penetrates the cell, it does not resemble the *in vivo* situation where H_2O_2 dissociates from mitochondria and reacts with surrounding cell components. Due to these facts, reaction of the cells to oxidative stress induced in this way might not reflect *in vivo* situation.

A way to circumvent these difficulties would be to use chemical compounds increasing free radical production in the mitochondria, for example menadione.

The experiments performed in this study concentrated on the effects of NT-3 and GDNF on only two antioxidant enzymes, catalase and SOD Cu/Zn. Both are important steps in the process of removal of free radicals. However, neither mitochondrial SOD Mn, nor glutathione enzymes were examined. It is possible that although NT-3 does not affect protein levels of catalase and SOD Cu/Zn, it might affect levels of SOD Mn, GPx or GR, at least upon prolonged incubation with NT-3. Since the protective effects of NT-3 were rapid, a likely explanation would be the effect of NT-3 on the activity of antioxidant enzymes present in the enteric ganglion cells since it might require less time than affecting *de novo* expression of enzyme molecules. Similar questions are also relevant in case of GDNF.

Due to the difficulty in obtaining sufficient amount of proteins for western blotting, cultures designed for protein extracts were not subjected to H_2O_2. One has to bear in mind, however,

that H_2O_2 exposure in itself is likely to initiate signalling events [Lai et al. 1996]. Considering this fact, the results gathered in this study, based on the changes of signalling pathways upon trophic factor stimulation might not reflect all signalling events that take place in the cells subjected to both NT-3 and H_2O_2. Addressing this issue would be a valuable addition to the experiments described in this work.

There are also quite interesting issues remaining to be answered, concerning the NT-3 initiated signalling events and resulting cellular responses. It is known that the duration of the trophic factor stimulus can result in different cellular responses, which might range from increasing of cell survival to initiation of cell death. In this study, only short term effects of NT-3 stimulation on signalling pathways were examined. It might be interesting to investigate whether prolonged NT-3 exposure would have different effects, especially in conjunction with H2O2 induced oxidative stress. Also, only few selected signalling molecules were examined. Investigating the effect of NT-3 stimulation on molecules directly involved in initiating cell survival, such as CREB, or cell death such as p53 or JNK might be a valuable complementation of the results of this study.

The result of this work, as well as result obtained by other groups suggest existence of functional interplay between PI-3K/Akt and MEK/MAPK pathways. Although not investigated in this study, such interplay might be crucial for determining final outcome of specific trophic factor stimulation, and as such might be important for devising strategies of preventing neuronal death occurring as effect of oxidative stress in vivo.

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